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PROCEEDINGS OF THE 3RD AQUATIC TOXICITY WORKSHOP

HELD IN HALIFAX, NOVA SCOTIA

NOVEMBER 2-3, 1976

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Atlantic Region

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PROCEEDINGS OF THE 3RD AQUATIC TOXICITY WORKSHOP, HELD IN HALIFAX, NOVA SCOTIA, NOVEMBER 2-3, 1976

EDITED BY

W.R. PARKER, E. PESSAH, P.G. WELLS, G.F. WESTLAKE

ENVIRONMENTAL PROTECTION SERVICE
HALIFAX, NOVA SCOTIA
CANADA

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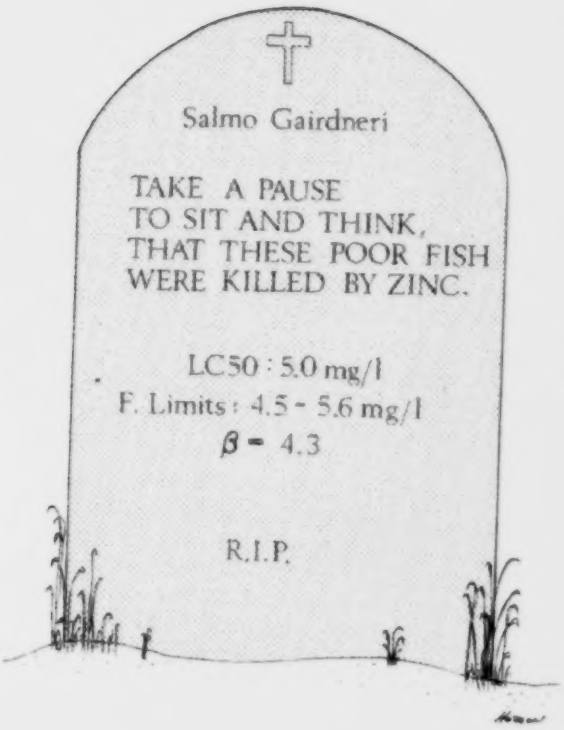
Salmo Gairdneri
TAKE A PAUSE
TO SIT AND THINK,
THAT THESE POOR FISH
WERE KILLED BY ZINC.
LC50 : 5.0 mg/l
F. Limits : 4.5 - 5.6 mg/l
β = 4.3
R.I.P.

SERVICE
TOXICITY EVALUATION SECTION

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PREFACE

The Environmental Protection Service (Atlantic Region) hosted the third Annual Aquatic Toxicity Workshop in Halifax in 1976. The aim of these workshops has been to exchange technical information on methods and applications of aquatic bioassays. The first workshop was held in Winnipeg by the Freshwater Institute in 1974 and the second in Toronto in 1975 by the Ontario Ministry of the Environment.

The 1976 proceedings include papers presented on both technical and philosophical aspects of the major theme of the workshop: 'Standardization and protocols of bioassays'. Also included are discussions and numerous question periods from the meetings. During the sessions, a number of participants suggested that standardization was desirable for bioassays used in regulatory work but not desirable for research. Some participants agreed that all bioassays should be related to solving specific environmental problems; others felt that investigating bioassay methods without immediate application was a worthwhile activity. All agreed that interdisciplinary and intergroup collaboration on projects would enhance the development of valuable approaches to aquatic toxicological problems.

We are indebted to the authors, reviewers, participants, and staff of the Toxicity Evaluation Section, EPS, Atlantic Region, for making the workshop a success. It is our hope that future workshops of this kind, by improving communication, will contribute to significant advances in assessing and controlling contaminants presently impinging on our aquatic resources.

The fourth annual workshop will be held in Vancouver, November 8-11. Inquiries may be addressed to Dr. J.C. Davis, Pacific Environment Institute, Vancouver, British Columbia, V7V 1N6.

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Halifax, Nova Scotia
May, 1977

PRÉFACE

Le Service de la protection de l'environnement (région de l'Atlantique) était l'hôte, à Halifax, du troisième colloque annuel du Comité d'étude sur la toxicité de l'eau. Ces rencontres se veulent des tribunes d'échange d'informations techniques sur les méthodes et les applications des essais biologiques. Le première réunion avait en lieu en 1974, à Winnipeg, sous l'égide de l'Institut des eaux douces et la deuxième, en 1975, sous celle du ministère de l'Environnement de l'Ontario.

Le comte rendu du colloque de 1976 reproduit les documents présentes qui traitent des aspects techniques et théoriques du thème central, la normalisation des essais biologiques. On y résume aussi les délibérations et les nombreuses questions soulevées durant les séances d'étude. Bon nombre de participants ont cherché a démontrer que la standardisation des essais biologiques est utile à la réglementation plutôt qu'aux activités de recherche. Certains participants étaient d'avis que ces analyses devraient chercher à resoudre des problèmes environnementaux spécifiques; d'autres y voyaient des avantages certains, mais hors du contexte des applications immédiates. Cependant, tous ce sont accordés à dire qu'une collaboration accrue entre les groupes et les disciplines pourrait faire naître des méthodes d'approche plus sûres aux problèmes de toxicité de l'eau.

Nous exprimons notre reconnaissance à tous les auteurs, réviseurs et participants ainsi qu'à tout le personnel de la Section de la toxicité de l'eau du SPE de la région de l'Atlantique, a qui nous devons le succès de cette réunion. Nous espérons que les prochaines rencontres, en stimulant les échanges, nous aideront à mieux évaluer et maîtriser la contamination de nos resources aquatiques.

Le quatrième atelier annuel se tiendra à Vancouver, du 8 au 11 Novembre. On est prié d'adresser toute demande de renseignements à J. C. Davis de l'Institute de l'Environ nement du Pacifique, Vancouver, B. C.

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Halifax, Nouvelle-Écosse
le mai, 1977

STANDARDIZATION AND PROTOCOLS OF BIOASSAYS - THEIR ROLE AND SIGNIFICANCE FOR MONITORING, RESEARCH AND REGULATORY USEAGE

JOHN C. DAVIS*

Davis, J.C. 1977. STANDARDIZATION AND PROTOCOLS OF BIOASSAYS - THEIR ROLE AND SIGNIFICANCE FOR MONITORING, RESEARCH AND REGULATORY USEAGE. Proc. 3rd Aquatic Toxicity Workshop, Halifax N.S. Nov. 2-3, 1976. Environmental Protection Service, Technical Report No. EPS-5-AR-77-1. Halifax, Canada. pp. 1-14.

This paper reviews the subject of bioassay procedures, their standardization and the use of protocols in bioassay methodology. Test types are discussed and test selection techniques described. It is stressed that the original objective in doing the test be carefully defined and the most practical test to suit that objective selected. The nature of standardization, techniques for standardization and the dangers of over-standardization are summarized. Over-standardization can result in inflexibility, stifle innovation and lead to impractical conditions making the tests, in some cases, irrelevant. Priorities for future work are discussed with emphasis on the need for receiving water tests and relevance of test procedures.

Davis, J.C. 1977. STANDARDIZATION AND PROTOCOLS OF BIOASSAYS - THEIR ROLE AND SIGNIFICANCE FOR MONITORING, RESEARCH AND REGULATORY USEAGE. Proc. 3rd Aquatic Toxicity Workshop, Halifax N.S. Nov. 2-3, 1976. Environmental Protection Service, Technical Report No. EPS-5-AR-77-1. Halifax, Canada. pp. 1-14.

Ce document passe en revue la méthodologie, la standardisation et le processus des essais biologiques. Il fait le point sur les types d'analyses et sur les techniques qui permettent de choisir les mieux appropriées. Il fait valoir la nécessité de bien définir l'objectif premier d'un essai pour ensuite choisir la méthode qui y convient le mieux. L'étude résume les aspects essentiels de la standardisation, les techniques qui y conduisent ainsi que les risques de la normalisation à outrance. Cette dernière peut entraîner une inflexibilité gênante, étouffer l'innovation et, dans certains cas, engendrer des conditions non réalistes qui invalident l'essai lui-même. L'étude s'intéresse à l'ordre prioritaire des travaux futurs et insiste sur la necéssité de mettre à jour les méthodes d'analyse de l'eau et d'assurer qu'elles sont appropriées.

1.1 INTRODUCTION

In environmental studies and pollution-oriented work, bioassays have been a traditional method of documenting the presence or absence of apparent deleterious effects of pollutants on living systems. In my mind, the term "bioassay" describes a test or "assay" of some sort where biological material responds (or fails to respond) in some way to a given test condition. Frequently, such tests are done according to some pre-determined procedure, often set out in detail, which for the purpose of this paper we will describe as a "protocol". This paper then, deals with the subject of bioassays and protocols, their structure and intricacies, with particular reference to test standardization and relevance.

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This review is both an exposition of the complexity of the topic, as well as a plea for the use of clear logic and common sense in choosing, applying, standardizing and interpreting bioassays. This subject has become so widely discussed and bioassays used for so many things, that it becomes highly confusing and often the subject of heated debate.

Herein I hope to summarize and clarify the topic and to incite discussion among workshop participants. The topic, I believe, is not complex when examined carefully.

1.2 BIOASSAY TEST TYPES, THEIR DEVELOPMENT AND CHOICE OF TESTS

Traditionally, much of our bioassay methodology evolved from pharmacology where tests of drug interactions with whole animals have been conducted for years. Indeed, much of our statistical knowledge of how to deal with toxicological data stems from pharmacological practice (Bliss 1935), and it was common to discuss toxicant levels in terms of "dosage" (e.g. LD50) implying administration orally or by injection (Litchfield and Wilcoxon 1949). More properly, we now have settled upon the determination of toxic "concentrations" for describing lethal levels of aquatic contaminants. The term LC50, describing the concentration lethal to 50 percent of organisms tested, is now widely adopted.

A variety of test types are now available to us for toxicity tests. I summarized these tests last year (Davis 1975a) and present that summary in Table 1.

ADVANTAGES	DISADVANTAGES	OPTIMUM USE
	STATIC TEST	
- SIMPLE, INEXPENSIVE - ECONOMICAL OR TOXICANT AND MANPOWER - WELL DOCUMENTED	- DETOXIFICATION POSSIBLE - WASTES ACCUMULATE - VOLATILES EASILY LOST	- TEST < 12 HOURS - COMPARATIVE TESTS
	STATIC WITH REPLACEMENT	
- SIMPLE, FAIRLY ACCURATE - FAIRLY ECONOMICAL OF TOXICANT	- REQUIRES REGULAR ATTENTION - ORGANISMS STRESSED DURING REPLACEMENT	- ROUTINE LC50'S - TESTS < 96 HOURS
	CONTINUOUS FLOW	
- OFTEN MOST ACCURATE - NO DETOXIFICATION OR WASTE BUILD-UP - GOOD METHODS AVAILABLE	- COMPLEX APPARATUS - HIGH TOXICANT USE* - SPACE REQUIRED	- RIGOROUS LC50'S - TESTS > 96 HOURS - FOR MAXIMUM ACCURACY
	IN SITU	
- INCLUDES NATURAL CONDITIONS - CAN USE LOCAL SPECIES - NO EFFLUENT REQUIRED	- DIFFICULT TO OBSERVE AND MONITOR - ORGANISMS STRESSED DURING OBSERVATIONS? - DIFFICULT TO CONTROL AND INTERPRET	- RECEIVING WATER SCREENING TEST - TESTING INTERACTIONS UNDER NATURAL CONDITIONS

*with industrial effluent samples

Table 1. Bioassay test types, their advantages, disadvantages and optimum use (after Davis 1975a).

Basically, we have a progression in complexity of test type available to us and our task is to select the type of test which best suits our needs. This can be done in a logical manner:

a) Accurately define our objective - what is the purpose of the test and what answer do we wish to obtain? Will we learn any more by using a more complex type of test?

b) Select the general type of test that best fulfills step #a. For example, if we wish to simply compare toxicity of two waste streams, a simple static test may suffice. If we wish to very accurately define an incipient toxicity threshold of a long-term nature, a continuous flow procedure will be desirable.

c) Select the test organism(s). Again this factor relates to step #a - what is our objective? Is the organism representative and of sufficient sensitivity to meet our objective?

d) Select the desired protocol for conducting the test - i.e. the detailed list of specifications for the test. Report procedures used, and all relevant observations. Is the protocol practical in this case?

e) Measure and report all pertinent water characteristics (e.g. Temperature, pH, Salinity, Dissolved Oxygen, Hardness, etc.).

f) Analyse according to accepted statistical protocols, e.g. confidence limits, slope functions, etc. (Sprague 1969).

g) When interpreting results, consider the limitations of the test. For example, a static test rigidly done under constant laboratory conditions may not necessarily be very useful in the "real world" with its dynamic regime of physical, chemical and biotic factors. Similarly, a test done with rainbow trout at 15°C may be of little use for predicting effects on benthic invertebrates in a system devoid of salmonids.

I believe that we are currently going through a transition in our approach to bioassay useage and interpretation. At one time we were happy with acute static tests that gave simple "quick and dirty" results. We had little information regarding subtle sublethal effects or the relevance of our test procedures. Of course now we are more enlightened - we have discovered the myriad of things that affect bioassay results such as hardness, temperature, fish loading density, species sensitivity, aeration, effluent collection and handling procedures, etc., and our protocol documents reflect this complexity (e.g. Standard Methods, 14th ed. 1976). Thus we can tend to overkill in our protocols by adopting techniques so rigorous and complex that they lack practicality. For example, our regulations for some emissions call for complex flow-through tests with numerous replications that tax our laboratories to the point of impossibility. The result is that sometimes we don't use the procedures that appear in the regulations and may lose credibility for failing to follow our own guidelines.

In all this, there is a great need for a compromise position. We need to balance our desire for maximum accuracy and relevance with the real practical concerns of loading density, effluent volume, test duration, problems caused by replication, effluent collection and storage etc. If we keep in mind the limitations of the bioassay test, it is evident that it cannot tell us enough in most instances to warrant using anything but the simplest, most expeditious test for routine regulatory or monitoring purposes. If we wish to pursue research or desire extreme accuracy then by all means we must use our most sophisticated methodology. Again, the issue is resolved by proper choice of test procedure at the outset of the work.

1.3 STANDARDIZATION - ITS IMPORTANCE IN BIOASSAYS

I think we all realize the value of standardization in bioassay methodology. Obviously, there is a vast choice of test procedures, test organisms, conditions accompanying the test, etc. Due to this variability, it is sometimes very difficult to compare toxicity

test results from the literature. Besides a need for conformity for comparative use, regulatory agencies have a special need of standardization. Some advantages of standardization could be summarized as follows:

a) Allows selection of one or more useful techniques for use by a variety of laboratories.
b) Facilitates comparison of results and increases usefulness of published data.
c) Increases accuracy of results by specifying and avoiding complicating factors (advantage of standardized protocol).
d) Allows repetition of test, if desired.
e) Easily initiated and run by a variety of personnel if procedure is well documented.
f) Legalistic advantage if procedures are accepted by the courts.
g) Useful for routine monitoring purposes.

Standardization can be achieved in a variety of ways. The obvious precursor to standardization is a thorough knowledge of the various chemical, physical and biological factors that affect bioassay results and methods of minimizing interfering factors (e.g. Sprague, 1969; 1970; 1971; Lee 1973). From this information, detailed protocol documents are devised such as those specified in Standard Methods (1976), A.S.T.M. documents, F.A.O. publications or the protocols of E.I.F.A.C. (European Inland Fisheries Advisory Committee). Standardization may be achieved by selection of standard test species such as rainbow trout, and the use of detailed technical guidelines for water temperature, hardness, fish loading density, etc.

One possible technique for standardizing fish stocks involves inclusion of reference toxicants in the test procedure (Davis and Hoos 1975a, b; La Roche *et al.* 1970; Marking 1966). This topic is controversial and it is beginning to appear as if the advantages of reference toxicants may be somewhat limited. Perhaps some of the workshop participants can shed some new light on this topic through their recent work. Davis (1975b) presented some thoughts on the usefulness and application of reference toxicants.

In summary, standardization can be achieved by:

a) Adoption of detailed test protocols which minimize or standardize disturbing effects.
b) Use of standard test species (choice of several desirable)
c) Selection of certain test types designed with a specific objective in mind.
d) Possibly, by use of reference toxicants or 'disease free' certified test animals.

1.4 THE DANGERS OF OVER-STANDARDIZATION

I think we all see the need for bioassay standardization and test protocols, however, there is a danger of over-standardization. At such a meeting as this, it is particularly evident that many approaches, test organisms, rationales, etc. are being applied to toxicity testing and that a whole range of tests are being conducted. There is a danger that over-standardization may stifle this innovative and creative work, particularly if powerful groups such as government agencies do not recognize work that does not coincide with their own priorities. In a way, we are in this dilemma having selected only rainbow trout as a standard test fish. We have experienced the rather ridiculous spectacle of people being asked to conduct salt water bioassays with *Salmo gairdneri* that are too young and possibly physiologically unable to adapt to full strength seawater. Similarly, if it is desirable to make some sort of assessment of the risk of a particular toxicant to a certain fish community, and that community has no salmonids in it, how useful is it to do the tests using rainbow trout? Furthermore, is it desirable to do the tests under physical and chemical conditions not equivalent to the receiving water in question? Here I think we have fallen into the trap of over-standardization and have lost our flexibility.

Another danger of over-standardization is that it may commit us to a course of action that may be inflexible. For example, if we devise regulations that stipulate continu-

ous-flow bioassays, then are we not bound to abide by those regulations in some instances when simpler tests may be equally useful, yet eminently more practical? In this instance, care must be exercised to see which procedures get written into law (regulations) and which ones remain as useful 'guidelines' (based on the current useage of 'guidelines' and 'regulations' in Canada).

There are a number of situations where use of a standardized 96 hr acute toxicity test is not appropriate. For example:

a) Substances with delayed toxicity.

Certain toxicants may not act or express themselves immediately but may require time before effects take place. For example, Hubschmann (1967) described a case of delayed copper mortality to crayfish where the lethal threshold was estimated as 10-15 days.

b) Substances with protracted toxicity over time.

Instead of not expressing itself until several days has elapsed, as above, a toxicant may continue to kill test animals over a long period. In this case there is a delay in the toxicity curve becoming assymptotic to the time axis and an incipient threshold is not defined at 96 hours. Sprague (1969) discussed in detail, the need to accurately describe the toxicity curve when determining threshold effects. Obviously, it is essential to insure this procedure is correctly followed when comparing toxicity values.

c) Bioaccumulative Substances.

Acute 96 hr toxicity tests are not suitable for substances which bioaccumulate. Lethal or sublethal effects may not occur until some time has elapsed and a high level of toxicant has accumulated in an organism. Alternatively, if bioaccumulation occurs via toxicant ingested in food organisms, the standardized protocol where test organisms are not fed during the test has little application to the 'real world'.

To me, what is required is a small, but flexible battery of tests and protocols that may be used with discretion and consideration of the situation at hand. In this way, we retain flexibility, allow for regional disparity in test conditions and special considerations, yet retain sophisticated procedures should we desire to use them.

In summary the dangers of over-standardization are:

a) Loss of relevance through poor choice of species.
b) Loss of applicability through choice of a protocol or test procedure that does not fit the situation (e.g. threshold exceeds 96 hrs).
c) Through a and b, an error in decision-making may result that is irreversible in terms of damage caused.
d) Ignorance of interactions as test may not consider interactions in receiving water.
e) Some danger of being locked into a procedure for regulatory or legal purposes.

1.5 THE FUTURE - THE RELEVANCE OF BIOASSAY TESTS AND PROTOCOLS TO THE ENVIRONMENT

The biggest challenge facing us as scientists is to ensure that our laboratory results are relevant and applicable to receiving waters. Only if we do this, do we stand any chance of making the correct decisions to protect our environment on a long-term basis. Emission standards are useful as they allow us to focus our attention on reducing pollutant discharge from point-sources by practising our engineering technology. Emission standards however, say nothing meaningful about what will happen in receiving waters where interactions between industrial wastes, biota (e.g. bioconcentration) and physical and chemical factors may occur.

One often wonders if the bioassay procedures we routinely use in the laboratory can be meaningfully applied to the field. Is the sensitivity of the procedure similar and can we get similar results to a given toxicant stress in the field as in the laboratory?

Recently (Davis *et al.* 1976; Greer 1976) we attempted to test the sensitivity of our sublethal test procedures for Pacific salmonids by anchoring our floating laboratory barge, L. PACIFICA, in the vicinity of a kraft pulp mill outfall at Crofton, B.C. (Figure 1).

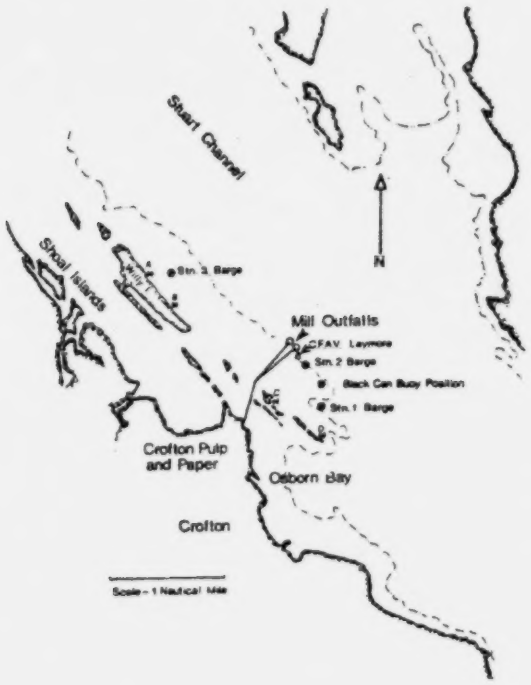

Fig. 1. The study area off the kraft pulp mill at Crofton, B.C., where sublethal effects of effluent on salmonids were described by Davis *et al.* 1976. High cough frequencies, reduced active oxygen consumption and avoidance behavior were noted in salmonids exposed to effluent-contaminated surface water at "Stn. 2 Barge". Little, in the way of sublethal effects was evident at Stations 1 and 3. Poor oyster condition factor was evident at shore stations B,C, & D.

The idea was that salmonids could be exposed to water pumped from a depth which contained little kraft mill effluent and their responses could then be compared with those in effluent-contaminated surface waters. At anchor station 2 (Figure 1), a high incidence in coughing frequency was evident in salt water-acclimated steelhead trout exposed to surface waters, particularly on ebbing tides when effluent moved past the barge (Figure 2). In addition, we observed avoidance behavior in coho underyearlings (Greer 1976) and altered maximal oxygen uptake rate in respirometer studies of the same species (Davis *et al.* 1976).

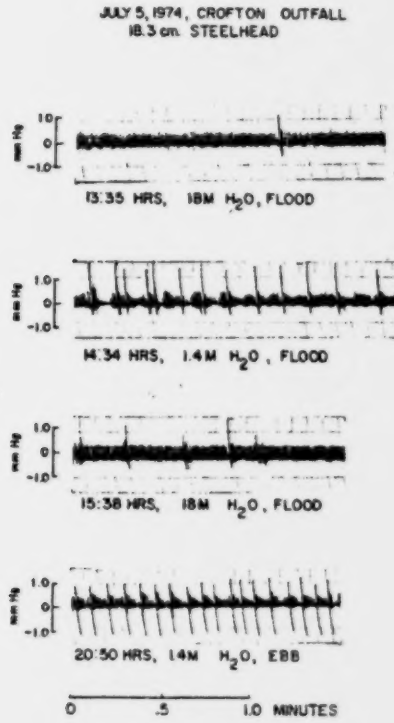

Fig. 2. Buccal pressure trace recorded from cannulated steelhead exposed to water from depth (18 m) or surface (1.4 m) at different phases of the tide. During the flood tide some effluent moved past L. PACIFICA but effluent presence in surface water was greatest on the ebb. Large spikes are 'coughs'.

Acute toxicity tests conducted daily using composite mill effluent yielded information on the toxicity of the effluent as discharged. Outbreaks of high toxicity appeared to correlate with observed sublethal effects in the field at Station 2.

A theoretical analysis of the toxic units expected to be present in the water was carried out (Table 2). The toxic unit concept (Sprague 1970) expresses the strength of a given toxicant as a fraction or proportion of its lethal threshold concentration. Thus 1.0 toxic units = the incipient LC50. Using various dilution factors and a range of LC50 values, it is possible to calculate the toxic stress expected for a given LC50 and dilution at the mill diffusers, assuming toxicity did not change in the environs of the outfall. Davis (1974, 1976) summarized the sublethal threshold values reported in the literature for salmonids and kraft mill waste. At 0.05 96 hr LC50 some sensitive sublethal effects may be present.

Table 2

Theoretical Dilution at diffuser	Effluent LC50 - % full strength				
	10	20	40	65	100
20:1 = .050	.50	.25	.125	.076	.05
30:1 = .033	.33	.167	.083	.051	.033
40:1 = .025	.25	.125	.063	.038	.025
50:1 = .020	.20	.10	.05	.031	.02
100:1 = .010	.10	.05	.025	.015	.01

$$\text{Toxic units} = \frac{\text{Conc. present in solution}}{\text{96 hr LC50}}$$

.05 toxic units = approximate threshold for most known sublethal responses of salmon to KME (Davis 1976).

Table 2. Theoretical calculation of toxic units present at the Crofton B.C. pulpmill outfall (body of table). Given a specific dilution and LC50, estimation of toxic units present (assuming no interaction with other substances in the water) is possible. The staggered line represents the transition zone between the presence or absence of sublethal effects - i.e. - to the left of the line effects are expected (0.05 T.U. or more) and to the right of the line few sublethal effects are expected (from Davis *et al.* 1976).

Thus, from Table 2, sublethal effects would have been expected at any portion of the table to the left of the staggered solid line. Based on our acute toxicity tests which indicated the effluent LC50 was often around 20% of full strength, and the work of Waldichuk (1964) who showed the dilution at the Crofton diffuser to average 22:1 and vary from 19:1 to 71:1, it would appear from Table 2 that between 0.25 and 0.05 toxic units were present at times during the study. The sublethal threshold for coughing responses, swimming stamina effects, oxygen uptake effects and avoidance lies in this general range of KME toxic units (Davis 1973, 1976; Greer 1976; Howard 1973; Walden 1976). It would appear then, that a similar sensitivity may have been observed in the field to that demonstrated in the laboratory. If this is true, it is encouraging as we may be able to apply laboratory results to such field situations.

I presented the above study as an example of the type of work that must go on to test pollutant effects upon receiving waters and aquatic organisms therein as well as in the laboratory. Our crude beginnings are full of weaknesses, and much more needs to be done to test procedures and develop new techniques, particularly in the marine environment. Indeed, we can never expect to have all the answers, but we must develop useful predictive tests.

I am convinced that acute and sublethal tests with aquatic organisms are only a part of the job of assessing the impact of pollutants on receiving waters. Ideally, we must understand effects at the community level, possibly via energy flow analysis in the communities. Sessile 'captive' organisms may prove to be especially useful indicators of pollutant effects, as they may be more sensitive than fish tests. For example, in our Crofton study we could only detect sublethal effects in salmonids within about 300 m of the pulp mill outfall. The oysters on the adjacent beaches, however, had low condition factor (relative fatness) up to 1.2 km from the outfall. Thus, a study of the organisms inhabiting the beach might, in the Crofton case, prove more useful than sublethal tests with fish. Indeed, 'captive' sessile organisms which can be studied by intertidal or benthic survey techniques may

frequently prove highly useful as indicators of pollutant effects. Similarly, zooplankton and phytoplankton tests may prove to be very useful as receiving water tests, particularly if they play a key role in the food chain leading to important commercial and recreational species. A great deal remains to be done to identify simple, sensitive and highly representative test organisms and test procedures for receiving water study.

1.6 REFERENCES

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1.7 QUESTION PERIOD

Ed Pessah, Environmental Protection Service, Halifax: John, in standardization we generally standardize certain test parameters, but only from the start of the actual test. We should give more attention to the standardization of important factors such as temperature, photoperiod, ration and density related to pre-test conditions. The pre-test conditions have a great potential to affect certain responses in various organisms. If we could standardize them, the differences observed between labs and between water qualities and so on would be negligible unless they deal with things that are very rigidly water quality dependent. At least then we would be in a much better position to separate out effects due to water quality and minimize our overall experimental 'error factor'. As a specific example a paper was presented at the ASTM conference last week which dealt with the quality of ration. A difference in the LC50 value in the order of 2-3 fold was elicited based on the quality of protein in the diet. Now I don't think we're going to get that kind of difference solely due to labs when we have that kind of difference due potentially to diet. Should we increase the research effort in that area or at least document some interim approximate figures for standardization of factors that are not of direct interest to the investigator (e.g. food quality and ration)? Would we then not have a better opportunity to make more significant contributions towards the understanding of the biology of the organisms with which we deal?

John Davis: Ed, I agree that we have to take a closer look at factors such as ration, nutritional quality of food, temperature, photoperiod, etc. and their effect on bioassay results. There is a good deal of information on this and much of the problem may be in getting people to agree on desirable levels of pre-test conditions. The aquaculture literature has some good information on high quality diets. Unfortunately, with things such as temperature there are regional differences that cannot be ignored. For example, 15°C may be useful as a test temperature for running bioassays in the central and eastern regions while on the west coast we are more confortable with 10°C. I think we have to recognize the need for some rerional flexibility in these details. In terms of identifying research priorities in support of protocol development, I think the role of workshops such as this should be to identify such priorities.

F.K. Fahmy, Beak Consultants, Mississauga: In your paper in the Journal of the Fisheries Research Board you mentioned that most sublethal effects were elicited at .05% of the 96-hour LC50 and you recommended .02% as a safe level. On what basis do you make this claim?

John Davis: The paper you refer to very clearly points out that most known sublethal effects of kraft mill waste on salmon appear to be absent at 0.05% of the 96 hr LC50. The paper does give examples of some sensitive sublethal responses at lower levels as well as voicing a concern over possible synergistic effects and the need for some degree of safety during

black liquor spill conditions. I therefore recommended the level of 0.02 LC50 as one which provides a good margin of safety, pointing out, that in some instances, this may be a difficult dilution to achieve.

If you are having problems with some people interpreting this paper in other ways, ask them to go back and carefully read the paper. It is purposely written in that manner to allow people to exercise judgement and flexibility in applying such recommendations to site-specific cases.

Cecil Innis, Ontario Ministry of the Environment, Rexdale: After the discussions on reference toxicants from our last meeting we embarked on a number of tests using phenol without aeration, etc. We were putting the cart before the horse. We didn't consider things like mercury levels in fish foods which actually exceed limits for Ontario. Also rainbow trout are treated with malachite green.

Gary Alexander, Freshwater Institute, Winnipeg: Would you elaborate on the use of sublethal and acute lethal tests in a sequence for the purpose of screening effluents, i.e. the protocols approach?

John Davis: I like the idea of having a sequence of tests available for effluent screening purposes. We could have a scheme where we first take a quick look at the effluent using some rapid test such as the residual 0_2 bioassay, GMST test or a similar procedure. This would give a rough indication of toxicity thresholds and approximate dilutions needed for bioassay and would estimate the magnitude of the problem - i.e. - is the effluent sufficiently toxic to warrant further testing? Then, it would be desirable to do a rigorous incipient LC50 test using good techniques to establish the acute toxicity. Also desirable would be some evidence on bioaccumulation potential of the effluent constituents and sublethal effects, if there are resident species threatened in the receiving waters. Bioaccumulation could best be approached by tissue analysis for body burden determination with an existent discharge or by long-term sublethal experimental exposures for new discharges. Regarding the choice of sublethal test methodology, I would favor tests related to behavior, growth, feeding and reproductive success, particularly, if organisms with short generation times could be employed. The choice as to how much or how little of this we would have to do for each effluent should be left flexible for consideration of each effluent on an individual basis.

Ed Pessah: Fisheries and Marine Service are about to come out with legislation governing the health of fish that can be sold. This should come into effect on January 1, 1977. There is a pathology group in F&MS in every region and I suggest we contact the regional pathology group of F&MS to get more information.

On Gary Alexander's point about screening effluents, we might look at the advisability of having a selection of representative test organisms that can be tested at various points in their life cycle. Using this approach, the most sensitive organism or the most sensitive stage in the life cycle of that organism could be identified as part of the screening process. Presumably one would start with acute lethal bioassays as the benchmark and proceed on a subacute and sublethal tests. The point is that one cannot often predict which organism or life stage will be most affected by any given toxicant. A selection of bioassay modes from acute lethal to chronic sublethal would be useful when superimposed on the basic screening test design.

Rick Hoos, Environmental Protection Service, Vancouver: Concerning the regulatory bioassays that are frequently etched in stone -- don't you think it might be a mistake to specify the test procedures in such detail, particularly after consideration of what we are trying to achieve with the test -- namely, whether an effluent passes or fails?

John Davis: I agree Rick, and I tried to make that point in my paper. Often being too specific may lock you into a procedure and result in difficulty. I think we have to, at least in terms of current Canadian useage, be very careful of what items we place in 'regulations' and 'guidelines'. Perhaps the toxicity criteria of a pass or fail nature should be placed in the regulations and the details of test procedures in the guidelines with some consideration for flexibility within those guidelines.

Gordon Craig, Ontario Ministry of the Environment, Rexdale: I agree that the transport of effluents over long distances in the quantities that are required for continuous-flow bioassays is impractical and therefore makes the Federal regulation unenforceable. What would you propose as a viable alternative for the continuous-flow test?

John Davis: With regard to pulp mill effluent bioassays, I see nothing wrong with static tests with replacement provided the guidelines for fish loading density and effluent handling procedures are followed carefully. In fact, several labs have shown that GMST technique or the rapid residual O_2 test is useful in estimating the 96 hr LC50 and possibly even for detecting sublethal levels of effect. Thus for routine monitoring purposes, I am in favor of the quickest, simplest, most economical test possible. If that test shows a real problem exists, then perhaps we need to adopt more rigorous tests for legalistic or research purposes.

Don McLeay, B.C. Research, Vancouver: With regard to your comment that static is inferior to batch replacement which is inferior to continuous-flow - we find that with pulp mill effluents when we do 24 hour batch replacement with 2 litres per gram of fish and compare it with a continuous-flow with the same loading and exchange rate, we get the same results. Therefore, I see considerable advantages to carrying out batch replacement bioassays in terms of effluent volume, labor and special equipment requirements.

Bob Wilson, Environmental Protection Service, Halifax: Earlier in your talk you mentioned that one should be careful to use static with replacement bioassays in the least stressful manner possible. Would you care to enlarge on that in terms of whether the procedure might have an effect on the outcome of the tests?

John Davis: In conducting static bioassays with replacement it is possible to stress the fish during the solution replacement procedure. Fish undergo considerable stress when the solution level in the tank falls, particularly if their dorsal fins break the surface. Similarly one must be careful to minimize visual stress while replacing solutions. A good way to do this is to have each tank set up with a siphon and filling hose so one half the volume can be drawn off and then new solution gently added. Even transferring fish from tank to tank in net baskets or handling nets risks stressing the fish. There is considerable evidence of physiological upsets such as osmoregulatory effects, hematological effects, altered liver and plasma glucose and glycogen levels and elevated blood corticosteroid levels following even brief periods of handling. All these physiological effects could have a major effect on toxicant uptake and mode of action and alter the result of a toxicity test.

Gary Alexander: In the case of an effluent where there are many different toxicants in one solution and the same effluent may differ from one day to another, wouldn't it be wise to use a test that covers all these possibilities?

John Davis: I think most of the toxicity tests will deal with the toxicity of mixtures of toxicants provided they are properly done using the receiving water as diluant water and are carried on long enough to cover possible long-term synergistic effects. The real problem comes with temporal variability in effluent composition. About all we can do is test lots of samples and try to get a feel for the 'average' toxicity. A 24 hr composite sample will help smooth out short term variability but collection of composite samples over longer

periods is probably unwise and prone to error. Another solution is to have 'indicator species' such as those used by Cairn's group 'on line' in the system and set up so they trigger an alarm during spill conditions according to their sublethal responses.

Ed Pessah: If we are confronted with oscillations in effluent quality, would we be better off to not do the lethality test since it does not permit you to gather any information on the recovery of the normal effluent quality. Is this not an excellent situation for using sublethal rather than lethal responses in that sublethal responses permit you to actually monitor the amplitude and duration of upset as well as generally being able to follow oscillation in effluent quality?

John Davis: That is a good point. We may be committed to the acute toxicity tests now due to the nature of our legislation and monitoring requirements. Even with a toxic stream it would be possible to dilute that stream and monitor variations in sublethal effects with time. One would have to be very careful of the effects of acclimation dampening the response of the sublethal test species. At least with pulp mill effluent, acclimation has been shown to diminish the strength of some sublethal responses with time.

Evan Birchard, Imperial Oil, Toronto: We had occasion to try out a new chemical on a particular process in one of our refineries and it became necessary for us to go through the newly-enacted Environmental Contaminants Act. We had the somewhat dubious distinction of being the first to come under the Act. Since there were no guidelines yet, we started to look at some of the novel approaches such as tests for carcinogenicity. Do you have any comment on these types of tests?

John Davis: I think we will see more of these types of tests coming, particularly with regard to effluents that may contain hydrocarbon-type contaminents. The work is well along in identifying tumor occurrence in bottom-dwelling fish off major sewer outfalls at various places in the world and may serve as an index in monitoring long-term changes in water quality in coastal bays and inlets. There is an active international group working in this area - for information I would suggest you contact Dr. Hans Stitch, Cancer Research, Faculty of Medicine, University of British Columbia, Vancouver,B.C.

Don McLeay: There are now rapid tests available for measuring possible mutagenic/carcinogenic effects of environmental contaminants stemming from some work in Berkeley. I believe Burlington is also involved in this area. One of them is called the Ames test (a bacteriological test) and there are some tissue culture tests for carcinogenicity.

I also have a point on reference toxicants. After last year's toxicity workshop we decided we were going to do weekly bioassays with pentachlorophenate to get some idea of the tolerance and condition of our fish stocks. We find that, at least for comparisons of fish condition within a single laboratory, where water quality is fairly consistent, the routine use of a reference toxicant is a very useful technique to assess the quality of our fish stocks.

Garth Fletcher, Memorial University, Newfoundland: Most fish probably have distinct annual physiological changes which could significantly alter the results of a bioassay. For example, we have been studying the annual changes in a number of blood parameters in a common inshore marine fish (winter flounder) and have observed a two-fold change in hemoglobin concentration. This would mean large changes in the ability of the blood to carry oxygen. Another point we have observed is that the effect of a stress (e.g. capture) on plasma electrolytes is 2-4 fold greater during the summer than at any other time of the year. This would suggest that the effects of other stressors such as toxicants would also change seasonally. This may have particular significance to field type bioassays.

With reference to rainbow trout, a number of years ago Holmes and Stainer showed that the urine flow and glomerular filtration rate change dramatically during a period of smoltification. These changes occurred in laboratory maintained fish at constant temperature. Do such physiological changes not demand more research into the test species and perhaps necessitate the use of a standard toxicant? If I may bring up another point, I believe phenol is a diuretic, therefore any annual change in kidney function could alter the response to such diuretics. If the unknown toxicant is not a diuretic the use of phenol as a reference may be misleading.

Another comment on diet and disease of stock populations. I believe there is information in the literature which suggests that the extracellular fluid space changes with the rate of growth. Therefore if you maintain your stock fish on different diets and so affect growth, the extracellular fluid space will also differ. Again, if the reference toxicant is a diuretic (phenol) differences in extracellular fluid volume could have an effect on the results. The same point could be made with reference to epithelial diseases which give the fish problems regulating body fluids.

John Davis: These are all excellent points and are aimed at the root of our problems - namely, trying to apply the results of our work to the real world where the effect of season, diet, age, temperature,etc. can have major effects on the physiology of the organism and the nature of a toxicant effect. To me, this is the area where we have to place our research effort, to ensure that our tests, standards and guidelines are truely meaningful in terms of protecting aquatic organisms from toxicant discharges.

STATISTICAL CONSIDERATIONS IN PLANNING AQUATIC BIOASSAYS

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Hodson, P.V., C.W. Ross, A.J. Niimi, and D.J. Spry.
1977. STATISTICAL CONSIDERATIONS IN PLANNING AQUATIC BIOASSAYS. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5-AR-77-1, Halifax, Canada. pp. 15-31.

Detailed understanding of the assumptions and statistical calculations of probit analysis are limited to a few statisticians. For the practitioner, probit analysis using computer programs is a "black box" method, i.e., the derivation of the results may not be subject to question. This paper examines the results of contrived and real toxicity data using a probit analysis program to illustrate how experimental design (e.g. number of concentrations, fish per tank, and replicates) affects fiducial limits, chi-square, slope and variance of slope. Recommendations are also made for experimental approaches that would provide a more rational bioassay data base to support legal and environmental requirements.

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Un petit nombre seulement de statisticiens possèdent une connaissance approfondie des hypothèses et calculs statistiques de l'analyse probit. Pour le technicien, cette méthode informatisée est souvent un procédé mystérieux, dont la dérivation des résultats ne saurait être mise en doute. La présente étude examine les résultats d'un programme d'analyse probit, appliqué à des données hypothétiques ou réelles, pour montrer comment les éléments du devis d'expérience (e.g. nombre de concentrations, de poissons par réservoir et de reprises de l'expérience) affectent les limites de confiance, la règle du χ^2, la pente et la variance de la pente. On y trouve également l'interprétation de ces paramètres biologiques et statistiques. Les auteurs recommandent certains devis d'expérience qui donneraient des renseignements plus rationnels pour appuyer les démarches juridiques et administratives.

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2.1 INTRODUCTION

A bioassay, in its widest sense, is "the measurement of the potency of any stimulus, physical, chemical, biological, physiological or psychological, by means of the reactions that it produces in living matter" (Finney, 1971).

In aquatic toxicology, acute lethal toxicity tests with fish or invertebrates are usually intended to assess the numerical value of toxicity, to compare potencies of toxicants, to assess the effect of environmental variables (e.g., pH, temperature) on toxicity, or to assess whether effluents meet guidelines. In each of these cases a statistical expression of toxicity is desirable.

In practice, a typical lethality test consists of placing groups of organisms in several concentrations of toxicant, usually in a geometric series (equal intervals on a logarithm scale), and measuring mortality over a specific time period. Data are quantal, that is, the animals either live or die.

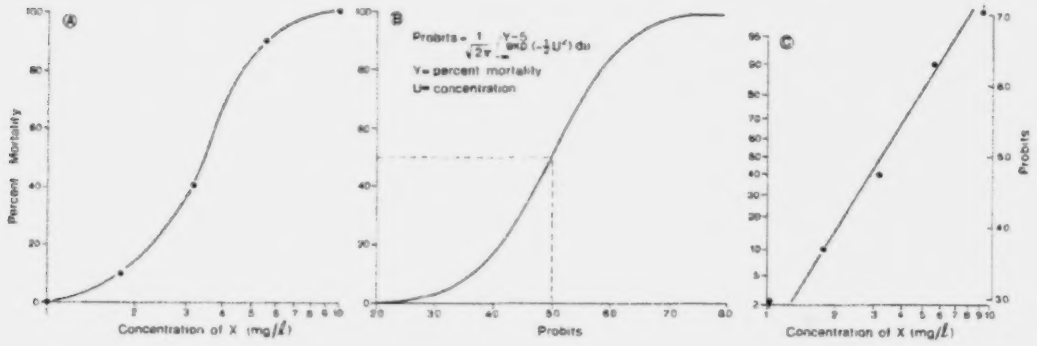

Fig. 1. A. The variation of cumulative percent mortality of a test population with the log of concentration of a hypothetical toxicant.

B. The relationship of probits to percent mortality.

C. The variation of probit of cumulative mortality with the log of concentration.

Generally, the proportions of test animals that die in a fixed time increase as the concentration of toxicant increases. The resulting graphs of percent mortality versus log of toxicant concentration produce a sigmoid curve (Figure 1A). Mortality of sensitive individuals accounts for the lower "tail" to the curve while highly resistant individuals account for the upper portion. The centre portion is almost linear and most representative of the average response rate of the test population. Consequently, estimates of the median lethal concentration, or LC50, of the toxicant are taken from this portion, and fiducial limits are calculated to describe the variability in this measurement. Fiducial limits are not the same as confidence limits (Finney 1971). The LC50 (or LC10, LC40, LC90, etc.) plus fiducial limits are most easily estimated by applying a transformation to percent mortality to create a linear relationship between mortality and log concentration. The most common transformation is the "probit" or "normal equivalent deviate". Percent response is related

to probits by a curve shown in Figure 1B. This curve is similar in shape to Figure 1A and the result is a straight line when probit of response is plotted versus log of concentration (Fig. 1C). Finney (1971) has published a complete statistical analysis of quantal data that involves - a) the probit transformation of percent mortality, b) the log transformation of concentration, c)the fitting of a straight line by a maximum likelihood or weighted least squares regression procedure and d) the estimation of the slope, LC50, and fiducial limits by calculation from the regression line and the error terms. These statistical procedures are not valid for non-quantal graded responses (e.g., survival time or percent inhibition of respiration, Finney 1971).

The mathematical derivations of these procedures are not easily understood and the computations are complex and lengthy using desk calculators. The result has been the development of simple nomographic techniques (e.g., Litchfield and Wilcoxon 1949) or computer versions of Finney's (1971) method. These methods have generally been accepted as "black boxes" into which data is entered and which generate LC50's, fiducial limits, slope, estimates of error on slope, predicted probits and a chi-square value for testing goodness of fit.

The purpose of this paper is not to re-examine the assumptions and derivations of Finney's probit analysis but rather to:

(a) show what factors in lethality test design that influence slope, fiducial limits and chi-square. Factors controlling the LC50's are the object of experimentation and will not be considered;

(b) make recommendations for experimental designs that would increase the precision of results of individual tests;

(c) illustrate the data requirements for answering environmental and legal questions; and

(d) make recommendations for experimental designs for data appropriate to these questions.

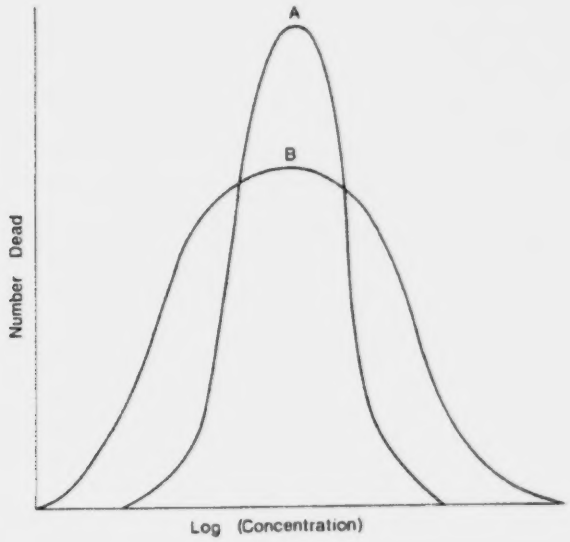

Fig. 2. Frequency distribution of mortality when the concentration of toxicant required to kill each specific organism is measured in two test populations.

Hypothetical data was used to illustrate specific points. By eliminating error, the hypothetical data permitted the test of single factors without confounding from other factors. The data was analyzed using a BMD03S probit program (University of California at Los Angeles) adapted to a CDC 3300 computer.

2.2 β - SLOPE

The slope describes the variation in Y for a given variation in X. In probit analyses the slope is expressed as probits/cycle, i.e., the probits of cumulative percent mortality for a 1 log cycle or 10-fold increase in concentration. The slope is an index of the range of sensitivity to the toxicant within the test sample of fish. A frequency distribution relating log of concentration required to kill each fish of a sample in a fixed time to the number dying at that concentration, gives a normal curve (Fig. 2). Curve A represents a population with a few sensitive individuals and a few resistant individuals, but with most responding within a narrow concentration range. The slope of curve A would be steep due to the sharp increase in percent mortality for a given increase in concentration. Curve B, by contrast, has many more sensitive and more resistant individuals. With this broader spread of sensitivity, a lower slope is achieved.

Slopes are not constant but vary among tests. This means that, biologically and statistically, the rate of change of mortality with concentration is not constant. Minimization of population variability and variability among slopes is a desirable feature of some lethality tests and has lead to the development of genetically uniform strains of fish. Other factors such as health, size, age and sex can also be controlled to reduce population variability and create a "white rat" test animal.

2.3 VARIANCE OF SLOPE

The variance estimate of a specific slope describes the uncertainty in the estimate of that slope. Since tests of parallelism may be used to compare tests, and since greater variance increases the likelihood that slopes will be judged equal, minimization of this variance is desirable. One cause of large variance is imbalance between the mid-point of the concentration range tested and the calculated LC50 (Finney 1971). This problem can usually be eliminated by using a geometric concentration series (equal intervals between log concentrations), as recommended by Finney (1971), and by testing an adequate range of concentrations based on preliminary tests. Fluctuating test conditions will cause fluctuations in the effect of concentration on mortality and hence in slopes.

2.4 FIDUCIAL LIMITS

The fiducial limits about an LC50 constitute a statement of probability. A 95% probability indicates that there is 1 chance in 20 that the LC50 does not fall within the specified limits. The interval between these limits is a measure of the precision with which we have measured a specific LC50 and is analagous to the precision limits of an analytical balance. Contrary to common practice, it is our opinion that fiducial limits should not be used as confidence limits to statistically compare LC50's, especially when fish populations are different or considerable time elapses between tests. The error terms used to calculate fiducial limits form the basis for tests of potency (the toxicity of one formulation versus the toxicity of a standard) (Finney, 1971).

The width of the fiducial interval is a linear function of the inverse of slope. Analysis of four probit plots, each with a different slope but the same LC50 (Figure 3A) indicates that increasing the slope decreases the fiducial interval (Figure 3B). In other

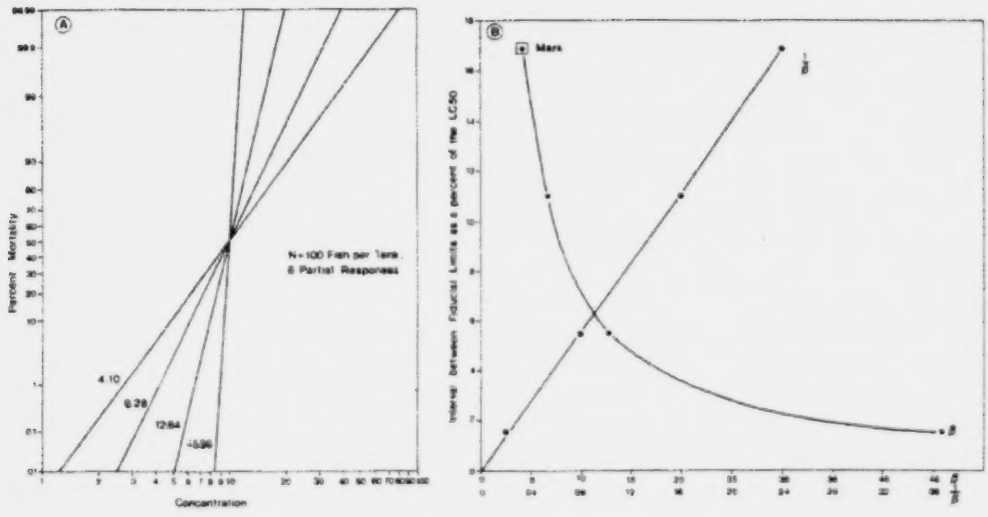

Fig. 3. A. Four hypothetical lethality tests with 100 fish per tank, 6 partial responses and the same LC50.

B. Variation of relative fiducial limits $\left(\frac{\text{Upper limit-lower limit}}{\text{LC50}} \times 100\right)$ with slope and the inverse of slope.

words, the population variability that controls slope also controls fiducial limits or precision. An increase of slope above 10-15 appears to reduce the interval only slightly.

The number of partial responses, i.e., the number of test concentrations giving between 0 and 100 percent mortality, also affects fiducial limits. For a probit plot of a given slope, an increase in the number of partial responses from 3 to 9 will produce approximately a 4-fold decrease in the fiducial interval (Fig. 4). This is also true even when the number of fish per test tank is reduced to permit more tanks at intermediate concentrations (e.g., if large numbers of small tanks are substituted for a few large tanks in a fixed space). Therefore to decrease the fiducial interval of a test, it appears necessary to increase the number of partial responses and/or the number of fish per tank. An arbitrary estimate of the optimal values would be about 5 partial responses and 20-40 fish per tank. This is based on the levelling out of the plane.

The decrease in fiducial interval, or increase in precision, with the number of fish per test tank is due to a decrease in the tabular "t" value used to calculate fiducial limits as well as a decrease in the standard error of the LC50 (Jensen, 1972) since N is the divisor for the calculation of standard error.

It should be recognized that the data from Figure 4 are based on a probit plot with a slope of 4.0. A steeper slope will lower the plane of response considerably; i.e., the relative intervals between the fiducial limits will be much smaller. There are some tests, especially those of effluents, in which partial responses are never observed, no matter how closely spaced the concentrations. Consequently, LC50's must be estimated by interpolation between the concentrations causing 0 and 100% mortality and no information is available on fiducial limits or slope. These data do not fit the model for probit

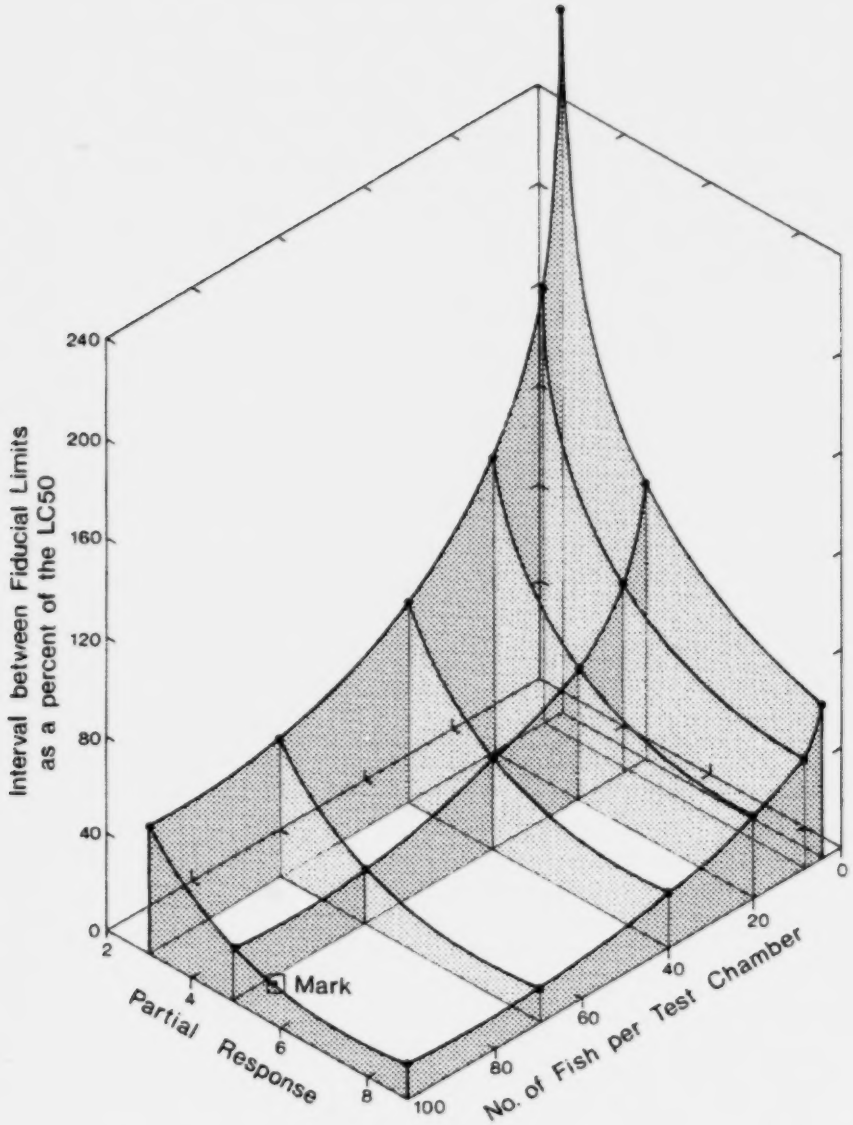

Fig. 4. Variation of relative fiducial limits ($\frac{\text{Upper limit-lower limit}}{\text{LC50}}$ X 100) with the number of partial responses and the number of fish per tank. The mark refers to the same data point in Figure 3. Results were generated from hypothetical tests with a slope of 4.0.

analyses and another, more appropriate analysis must be found. An alternative is to measure the mean survival (MST) of the test population at a specific lethal concentration. Calculations of MST's are not the subject of this paper but nomographic solutions are available (Litchfield 1949).

Variability in width of fiducial limits between tests may be attributable to differences in slope, i.e., some LC50's may be measured with a different precision than others due to a difference in the response rate of the sample of fish to the toxicant. For a pure toxicant, this suggests a change in the fish population or test conditions between bioassays. In this case, LC50's with abnormally low or high fiducial limits should be rejected, based either on a statistical comparison or the biologists" experience and judgement. If the toxicant is a mixed industrial effluent, a change in precision may be due to the composition of the mixture. Since this is a determinant of overall toxicity, the experimenter would probably retain the data.

If large differences in fiducial intervals are accompanied by similar slopes, the problem may be due to a change in the number of partial responses or an increased scatter about the line. In the first case, the data may still be acceptable. In the second case, the probit plot will have a larger χ^2 value. Acceptance or rejection of data will depend on the extent of the decreased precision when the fiducial limits are recalculated to recognize this scatter (see section about χ^2).

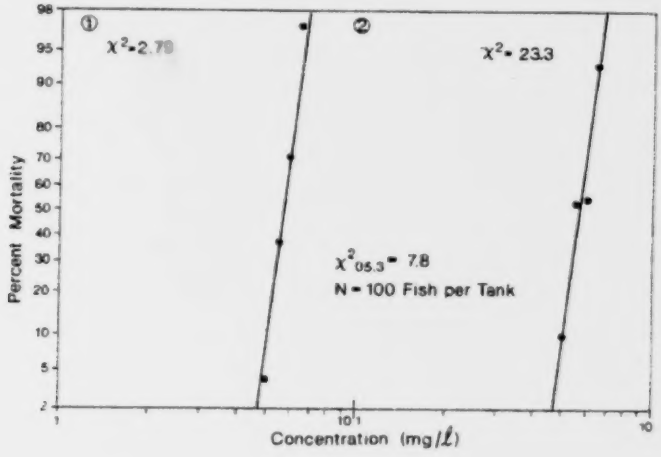

Fig. 5. The effect of location of point scatter on "goodness of fit". Line (1) "scatter at the ends", and line (2) "scatter at the middle" have the same slope and LC50 and are based on hypothetical data.

2.5 CHI-SQUARE

The χ^2 is the sum of the squares of the deviations of the observed points from the fitted probit plot and it indicates "goodness of fit". Increased scatter about the "best fit" line produces less confidence in the LC50. In probit analysis, observations close to 50% mortality are given more weight so that a deviation from the line near 50% mortality has a much greater effect on χ^2 than deviations near the ends (Figure 5). The χ^2 line for line #2, scatter at the middle, is unacceptable according to a table of χ^2 distribution. For line #1, scatter at the ends, χ^2 is much lower and is acceptable.

The number of fish per tank in a test also affects χ^2. When the χ^2 for lines 1 and 2 are recalculated as N decreases from 100 to 10 fish/tank, χ^2 decreases dramatically (Figure 6). These results indicate that our statistical expectation of a good fit increases with N and, if the fit is not better as in this hypothetical case, increasing N will increase the χ^2. Realistically however, we should observe a better fit when more

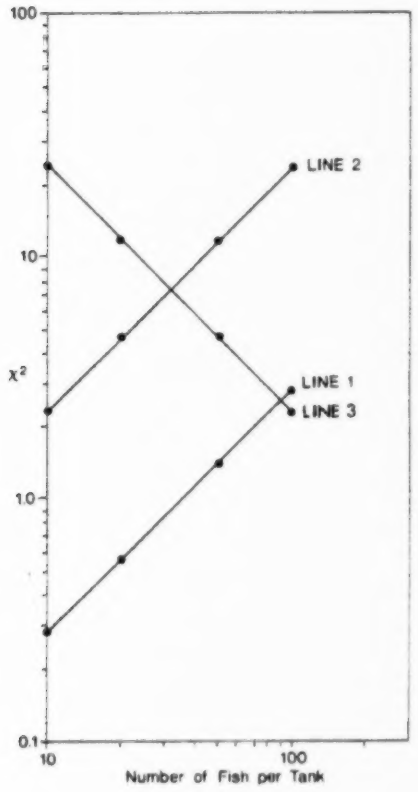

Fig. 6. The effect of number of fish per tank on χ^2 for lines (1) and (2) of Figure 5. Slopes, LC50's, number of partial responses, and deviations from the best-fit line were held constant. Line (3) illustrates the expected result of increasing N - i.e. smaller deviation from the best-fit line and a lower χ^2.

fish are tested (line 3, Figure 6) since unexpected random deaths will cause a smaller error in cumulative percent mortality and smaller deviations from the best-fit line. An increase in the number of partial responses should similarily produce a better fit, and, if this is not the case, χ^2 will increase dramatically.

Biologically, a significant χ^2 value does not always necessitate rejection of the data. If there is a consistent deviation from linearity (Figure 7B), the experimenter should re-examine the methods used since this variability may be attributed to experimental error (for example, a change in exposure conditions part-way through the test). In this case, data might be rejected, the cause of the deviation identified, and the experiment repeated. When variation about the line is random (Figure 7A), the fiducial limits about the LC50 should be recalculated to compensate for the scatter indicated by the χ^2. This recalculation involves multiplying the variance of the LC50 by a heterogeneity factor (H) equal to the χ^2 divided by the degrees of freedom ($H = \chi^2/df$) (Finney 1971).

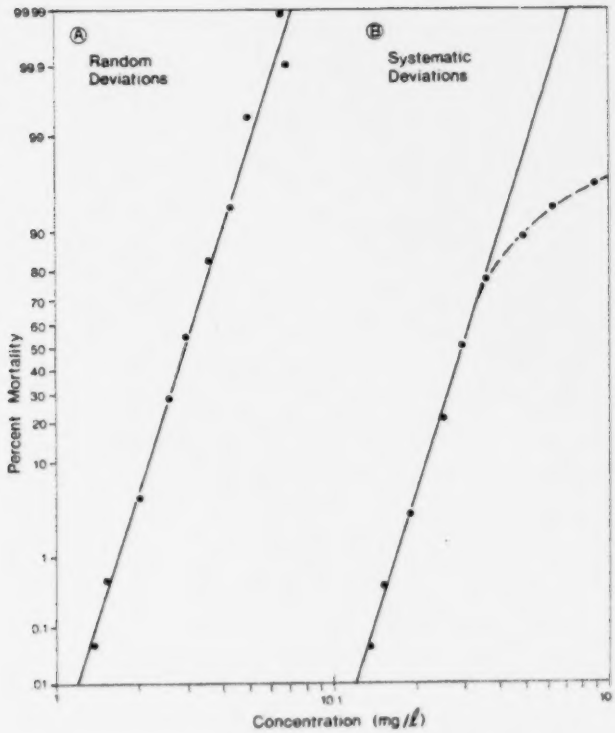

Fig. 7. Probit plots illustrating deviations from a straight line due to random error (A) or systematic error (B).

A new, wider set of fiducial limits is calculated using this new variance and use of the heterogeneity factor should be reported with the results. Therefore, one of the prime purposes of χ^2 is to alert the experimenter to systematic anomalies in his data and to allow computation of a realistic fiducial interval when scatter is random.

2.6 SUMMARY

In summary, an ideal test to estimate the LC50 of a toxicant should meet the following requirements (based on arbitrary judgements from the figures):

(1) Test at least 30 fish per tank in tanks of adequate size and with adequate flow rates;

(2) narrow the concentration range to provide at least 5 partial responses;

(3) select a range of concentrations such that the LC50 falls midway in the range;

(4) select a population of test animals of uniform health that provide a consistent response with very little difference among the more sensitive and resistant individuals; and

(5) ensure that exposure conditions are uniform and constant so that the slope of response remains constant.

2.7 REPLICATION

Obviously, the above requirements are stringent and may be difficult to meet due to physical constraints. Does the most precise test provide the best LC50? In our opinion, for environmental and legal questions, repetition of less precise lethality tests to give population responses would be far more useful. These would include a mean LC50, a standard deviation and 95% confidence limits. Note that fiducial limits were based on probit analysis while confidence limits are based on the mean and standard deviation of a normal distribution. If the repetitions are conducted within a short space of time (e.g. < 2 months), the standard deviation and confidence limits will give an estimate of the experimental error (error due to the measurement of the response) and sample error (error due to variations in the sensitivity of that particular population of test animals). If the tests are carried out over a longer period of time, space (among labs), fish stocks, etc., the variance may be partitioned to give estimates of the error due to these factors. A similar principle may be applied to tests of potency. While a single potency determination compares two single bioassays, population estimates of potency, based upon a number of separate comparisons, will be most valid for statistical descriptions of potency.

How then can the biologist best use his resources to meet the demands for toxicological data? The first step would be to accurately define the best model to answer the question posed by management. For example, the question might be whether a toxicant causes 50% mortality to a test population in a given period of time at a required concentration. Determination of an LC50 necessitates a test with 6-10 concentrations to give one estimate of whether the toxicant passed or failed. A better model would be to test six groups of animals at the required concentration and calculate the mean percent mortality with confidence limits. Pass or fail would depend on whether 50% mortality fell within the 95% confidence limits.

If the tests are to compare results between labs or to measure the absolute toxicity of a pure formulation, a good model would measure an LC50 under the most precise conditions possible, as described above, using a standard test species of high quality. Alternatively, if the toxicity of a known effluent in a particular stream is required, tests of that effluent on an appropriate test population, in the stream waters, would be best. Testing a salmonid in a stream where they do not occur would be highly unrealistic. Where salmonids are stocked as the native population, they could be obtained direct from the hatchery.

Therefore, the questions to be answered by the test predetermine the experimental model to be used and the constraints under which the biologist operates. How then can resources be optimized?

The first consideration should be that all lethality tests meet the requirements for oxygen concentration, fish density, flow rate, etc. (Sprague, 1969), so that these factors do not confound the results.

Second, the number of test animals per chamber must be large enough that chance mortality does not contribute excessively to error. For example, 1 chance mortality gives 20% error among 5 fish, but only 5% among 20. From Figure 4, our arbitrary guess is that five fish are an absolute minimum and at least 10 are desirable. If the alternatives are either one replicate with large tanks and 50 fish per tank, or 5 replicates with smaller tanks and 10 fish per tank, the second alternative would be best since there are 5 estimates of the LC50. Similarly, the statistical advantages of replication would be lost if data from five tests was combined for one calculation of an LC50. Attempting to produce a more precise test with a large N by combining the results of a number of smaller tests has a doubtful validity. This procedure also prevents the estimation of error between tests.

Third, the number of test concentrations giving partial responses should be a minimum of three if the advantages of computer analysis are to be realized and if LC50's are to be determined with reasonable precision. Larger numbers of partial responses will give greater precision, as shown in Figure 5, but if this precision is gained by sacrificing replication, it may be less worthwhile. For example, dividing a concentration range into 10 to give 6 partial responses and 3 reps for a total of 30 tanks would not be as efficient as dividing it by 6 to give 3 partial responses and 5 reps.

For a statistical treatment of lethality test results, what number of replications is adequate? The minimum number is three, to meet the basic mathematical requirement for calculating a standard deviation and confidence limits. Beyond this, the number of reps is dependent upon the size of the difference you wish to declare as significant and the expected variance of the measurement. For example, suppose an effluent guideline requires that no more than 50% mortality occurs within 96 hours at a specific concentration. You wish to say that in a sample of fish tested at that concentration, 60% mortality constitutes a statistically significant violation of the guideline. Because you are interested only in mortality greater than 50%, a one-tailed test is chosen. Assume a standard deviation, based on your previous experiments, of 5% mortality. The following formula may be used:

$$r \leq \frac{2(t_o + t_i)^2 s^2}{\delta^2}$$ (Steel and Torrie 1960)

where

- r is the number of replicates required;
- s is the standard deviation (= 5);
- δ is the difference we wish to detect in the parameter measured (60 - 50 = 10);
- t_o is the tabular t value associated with Type I error* at a given probability level for a one-sided test;
- t_i is the tabular t value associated with Type II error** at a probability level of 2 (1-P) for a one-sided test.

To use the formula, make a rough guess of r, say 10.

$$t_{o_{.05,9}} = 1.833$$

The desired probability of assuring the detection of a true difference of this size might be 90% (arbitrary choice).

$$t_{i_{(2\ (1-.90)),9}} = t_{.2,9} = .883$$

$$\therefore r \geq \frac{2\ (1.833 + .883)^2\ 5^2}{10^2}$$

$$\geq 3.7 \text{ rounded to } \underline{4}.$$

Repeating this process with the more realistic value of r gives a final r of 5.

*Type I error: experimenter rejects the null hypothesis when it is true (the effluent is declared too toxic when it is not).

**Type II error: experimenter accepts the null hypothesis when the opposite is true (the effluent is declared non-toxic when it is toxic).

Often a requirement for numerous replicates necessitates excessive time and effort. If the chosen model is the best way to answer the question, the biologist must either increase the size of the difference required to pass or fail the test, reduce the confidence level, or improve his technique to reduce the variance.

Is replication really necessary and practical? The following experiment may be used as an example. Lethality tests were conducted to determine the toxicity of various selenium compounds to zebrafish larvae (Brachydanio rerio) and were reported in detail by Niimi and LaHam (1976). Larval fish in groups of 50 were exposed to several concentrations of selenium d-l cystine. This procedure was repeated 10 times, each replicate having larvae from a different parent. All replicates were started within 10 days. The results were analyzed by probit analysis as separate replicates and as combined data.

The analysis of separate replicates produced 10 sample estimates of LC50's, χ^2, and fiducial limits. From these data, distributions of LC50's with means, standard deviations, and confidence limits were calculated to estimate population statistics. When the data were combined to give 500 fish/concentration, one LC50, with a slope and fiducial limits, was calculated to give a combined sample estimate.

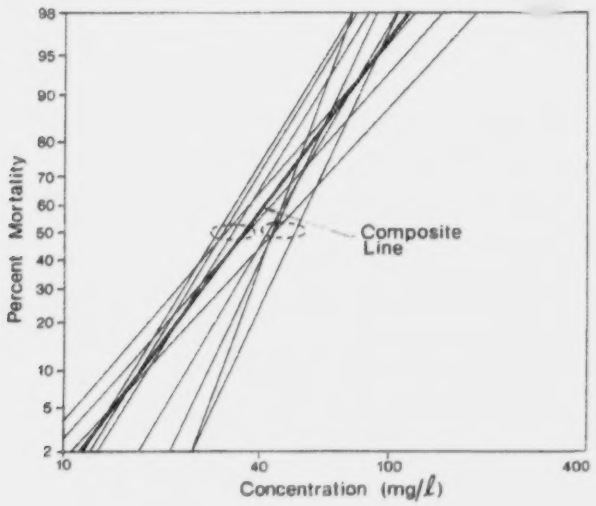

Fig. 8. Fitted probit plots of 10 tests of selenium d-l cystine using 50 zebrafish (Brachydanio rerio) larvae per test chamber. The heavy line represents a statistical analysis when data were combined artificially to give 500 larvae per test chamber.

Figure 8 shows probit plots of the 10 individual sample estimates and the single combined sample estimate of selenium d-l cystine. Slopes appear similar with some variability among LC50's. The combined estimate appears to be an average of these parameters. However, the frequency distribution of individual LC50's shows the need for population estimates (Figure 9). The distribution is clearly bimodal and the LC50's are grouped so tightly that the means are significantly different and have separate non-overlapping confidence intervals. Clearly, a single test of the toxicity of this compound would not

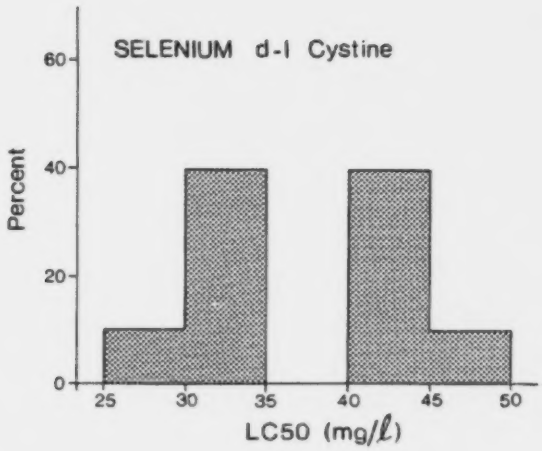

Fig. 9. Frequency distributions of LC50's from Figure 8. The means and 95% confidence limits were 31.1 ± 3.5 and 45.8 ± 10.0 mg/l respectively. The composite LC50 was 35.6 mg/l with fiducial limits of 33.8-37.5 mg/l. (LC50's: 29.5; 30.7; 31.0; 31.2; 33.4; 42.5; 44.3; 44.5; 44.9; 50.0)

properly estimate the population LC50, and a combined approach ignores the bimodal nature of the data. In addition, replication has provided an indication of repeatability that cannot be acquired from the combined approach.

It is also interesting to note that the fiducial limits of the combined LC50 do not include any of the LC50's of the individual replicates. However, the means of each of the two distributions have confidence limits that do include the values of each group. In addition, the fiducial limits of some individual tests within each distribution overlap the limits of tests in the other distribution. In other words, fiducial limits and confidence limits do not lead to the same conclusion.

Therefore, a mean of several LC50's with confidence intervals would be more valid statistically and biologically. However, the numbers making up the mean should be estimated with the same precision if statistical comparisons are to be valid. Fiducial intervals and slopes must be examined as previously described and acceptance or rejection of individual LC50's will depend on statistical and biological considerations.

2.8 CONCLUSION

In conclusion, it is clear that within the limitations of time and facilities, effort should be made to replicate acute lethality tests. Where facilities are limited, this will necessitate either repetition of the test over time, or reduction in the resources devoted to each rep. However, this is not a licence for poorly done tests; certain minimum requirements must always be met, and it is up to the biologist to make a realistic compromise between the precision on each test and the amount of replication and precision on the population estimates. Where resources do not allow both objectives to be met simultaneously, the question should be rephrased, the level of resources applied to the problem increased, or the tests postponed until resources are adequate.

It is not the intent of the authors to advocate hard and fast rules for number of animals per chamber, experimental conditions, number of reps, etc. The foregoing discussion was merely to indicate how these factors affect the quality and utility of results. The specific recommendations must come from those who design experiments or write guidelines and considerable thought must be given to the questions to be answered, the nature of the experimental material, and the limitations of resources.

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2.10 QUESTION PERIOD

Peter Doudoroff: In Figure 9, you suggested a biomodal distribution, but it looks as though it is a big blank missing out of a normal distribution.

Peter Hodson: The observed distribution was based on 10 replications. Thirty replications might have produced a different distribution. However the purpose of the graph was to show that one replicate does not represent the true situation. For this example, we do not have a good biological explanation for the biomodal distribution. It could be genetic, test methods, chemical transformation of selenium, etc.

John Davis: I was not quite sure by what criteria you were advocating comparing LC50's.

Peter Hodson: Replicate treatments using a good experimental design and test differences between mean LC50's by standard statistical methods. In contrast, Dr. Charles Stephan, U.S.E.P.A., feels that individual fiducial limits are a sufficient basis for comparing LC50's when they are based on the same stock of fish in the same lab and they are measured over a brief time period.

Gordon Craig: The work described by Finney used data on the response of beetles to an insecticide. It was therefore easy to cram any number of insects into a jar and increase the number of organisms exposed. It is not easy to increase the number of fish without affecting or allowing for increased loading rates. Most laboratories by convention choose equal interval log concentrations to run bioassays but the response of fish to such effluent concentrations can be quantal between adjacent concentrations. If one is striving to estimate an LC50 and is therefore obliged to calculate fiducial limits at least three partial responses are required. With this requirement, if conventional concentrations do not provide the appropriate response, closer concentrations must be established. Assuming a 10% error in measuring a concentration (see Zitko paper) and assuming a biological

response variation, there is a limit with which one can set up two concentrations which are intended to be different and do not produce similar responses. The question is, how close can one concentration be to another, and that is of course dependent on the two variations mentioned above?

Peter Hodson: The spacing between concentrations depends on the questions to be answered. If you wish one very precise measure of the LC50, choose a small interval between concentrations to increase the number of partial responses. Where facilities are limited and the choice is between replication or small intervals, I would prefer more replication with a greater interval between concentrations.

Dr. V. Zitko: How does the accuracy of the chemical analyses of concentrations affect your reasoning?

Peter Hodson: Finney's probit analysis model requires that concentration be an independent variable measured with no error. In aquatic bioassays, the concentration does vary but we assume that the mean concentration is representative. Where error is a significant proportion of the mean, the model may not apply and you should look for an alternative, perhaps a model based on covariance.

If you are trying to discriminate, by bioassays, the difference between 50 and 51 mg/l, and your chemical analyses are not that accurate, then conclusions based on bioassays will be invalid.

Bob Wilson: If you determine an LC50 by probit analysis you are getting an estimate of central tendency. If you take a number of averages from the same population then statistically you have to have a normal distribution. Did the graph you showed with the right and left handed L's say something about, as you suggest, biomodal distribution or the number of replicates required to demonstrate a normal distribution?

Peter Hodson: Yes, to both questions. However, more importantly it illustrates that one LC50 is only one estimate from a population of LC50's. Consequently one LC50 may not give a true representation of the mean. Therefore decisions based on the size of an LC50 should probably be based on the mean of several estimates.

Gary Alexander: Given that an effluent can produce partial mortalities, is there a theoretical foundation for using a single transformation such as the probit to calculate the LC50 of an effluent when multiple processes may be occurring?

Peter Hodson: The probit model can be used when number of animals dying follows a normal curve when compared to log concentration (Fig. 2). If this is true when multiple processes are occurring then the model is valid. If not, a new model must be formulated involving different transformations. Since most bioassay data are too sparse to allow construction of a normal curve, the best indications that the probit is not the correct transformation will be deviations from linearity in the probit plot.

John Sprague: A lot of people have brought up the problem that one may test 10% of something like pulpmill effluent and have no mortality but with 20% you have complete mortality with no partial mortalities in between. We all go into a tailspin because we can't calculate confidence limits. Frankly I never get very worried about this because you really have a very good bioassay since you know that the LC50 lies somewhere between 10 and 20%. In this case one can perhaps do without confidence limits for most practical purposes. It is actually a fairly precise test.

Peter Hodson: These data cannot be analyzed by Finney's method due to a lack of partial responses. However, there is no doubt that the data in this case are a good representation of the effluent's toxicity. Unfortunately, if a comparison of two effluents is required, and both give the same result (i.e. 0% mortality at one concentration and 100% at the next highest), then no comparison can be made despite considerable effort. A more efficient alternative would be to measure the mean or median survival time of fish at the same lethal concentration. This then provides a good mathematical basis for comparing toxicity.

Gordon Craig: When you are talking about replication in concentration have you done this with a toxicant? Measuring your responses, what variation in response do you get using your hatchery, etc?

Peter Hodson: Bioassays of zinc with rainbow trout at 15C produced the following results:

Bioassay #	Date	Fish Age (months)	Year Class	# of Fish Per Tank	Rep #	LC50 (mg/l	Fiducial Limits
1	10/08/76	≈30	73/74	5	1	1.00	.61-1.63
					2	1.77	1.09-2.88
					3	1.61	.98-2.63
2	1/11/76	10-12	75/76	10	1	.79	.62-1.01
					2	.31	.18- .54
					3	.32	.20- .50

The results indicate a marked change between the two test populations, perhaps due to age, size, testing time, or year class. Within a test population, the results are somewhat more homogenous.

Howard McCormick: Possibly you have read a recent publication in the Fisheries Research Board Journal by Spehar of our laboratory in Duluth, working with zinc and flagfish, and in this study he found a considerable difference in the threshold level depending on whether his fish were exposed as an embryo or a larval fish. I think it is important therefore to keep in mind the question you are trying to answer. If you are dealing with an environment the embryos are being exposed you may want to start there.

Peter Hodson: I couldn't agree with you more!

C. Vithayasai: A major contribution of this paper is to remind us of the replication principle and the associated concept of experimental error variance in the context of aquatic bioassays. Statistical textbook treatment of bioassay methodology places undue emphasis on the single experiment and negligently fails to exhort the user to follow conventional principles of scientific experimentation. Overwhelmed and brainwashed by such books, most of us are unable to rise above the probit mystique and see the LD-50 (or LC-50, or whatever) as simply a poorly estimated replicate mean.

The selenium D-l cystine replicated experiment on larval zebrafish provides a dramatic illustration of the relative magnitude of within - and between - replicate components of error variance in LC-50's, and perhaps merits relatively more comment. Parental source of larvae represented but one of the factors confounded with replicates and with additional background information this example might have further served to demonstrate the necessarily arbitrary nature of replication, and the consequent need for specific

details in "materials and methods" sections. The scope of statistical inferences is indeed broadened by replication, but only to that range of conditions which in some sense is covered by the replication.

Aside from this commendable review of the need and nature of replication, the paper appears to only contribute further to the mystique of probit analysis. A critical review of the applicability of this model in aquatic toxicity work might be more appropriate than some rule-of-thumb guidelines in its use. Growing evidence of the relationship between logarithmic MST and standard deviation favors the toxicity curves over the 96 hr LC-50, and points to an imcompatibility between the lognormal versions of these two models.

Peter Hodson: The comments on the probit "mystique" are quite appropriate. However, the removal of this mystique requires a simple explanation of the derivation of the method, its application, and the interpretation of results; both biologically and statistically. We are at a point that requires the services of a good statistician-biologist who understands probit analysis and its requirements for experimental design and can explain it to non-statisticians. The old "cook-book" approach will no longer provide adequate insight for solving new problems.

BEHAVIOURAL ASSAYS - PRINCIPLES, RESULTS AND PROBLEMS

EBERHARDT SCHERER*

Scherer, E. 1977. BEHAVIOURAL ASSAYS - PRINCIPLES, RESULTS AND PROBLEMS. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5AR-77-1, Halifax, Canada, pp. 33-40.

Behaviour is based on integration of underlying physiological functions (e.g. sensory processes, metabolism, hormonal conditions). Sublethal impairment at any of these physiological sites is likely to show up in the behavioural output. Some behavioural effects, particularly inter- and intraspecific responses, are difficult to demonstrate and quantify under standardized conditions. Certain locomotor reactions controlled by abiotic factors appear more suitable for routine testing. A fish or invertebrate may, in a quantifiable way, respond to a pollutant by approach or withdrawal (preference/avoidance tests), or by change of a given locomotor pattern (e.g. locomotor activity). The present state of using such responses in bioassays is reviewed, considering technical aspects and questions of interpretation. Options and conditions for standardization are presented and discussed.

Scherer, E. 1977. BEHAVIOURAL ASSAYS - PRINCIPLES, RESULTS AND PROBLEMS. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5AR-77-1, Halifax, Canada, pp. 33-40.

Le comportement, qui est la réaction globale de l'animal, part de l'intégration de certaines fonctions physiologiques sous-jacentes (e.g. les processus sensoriels, le métabolisme et l'état hormonal). L'atteinte toxique, non mortelle, de certaines de ces fonctions paraît vraisemblablement dans les manifestations du comportement. Certains effets éthologiques et, particulièrement, les reactions inter- et intraspécifiques sont difficiles à démontrer et à quantifier dans un ensemble de conditions normalisees. Pour les essais de routine, certaines réactions de locomotion déclenchées par des facteurs abiotiques paraissent mieux convenir. Un poisson ou un invertébré peut montrer une réaction quantifiable d'attirance ou de repulsion en presence d'un polluant ou manifester une modification des habitudes de locomotion, d'une certaine activité motrice par exemple. L'étude examine l'état actuel des procédés d'essais biologiques qui se fondent sur ce type de réaction, se préoccupant, en particulier, des aspects techniques et interprétatifs. Elle présente et analyse les options et conditions favorisant la standardisation.

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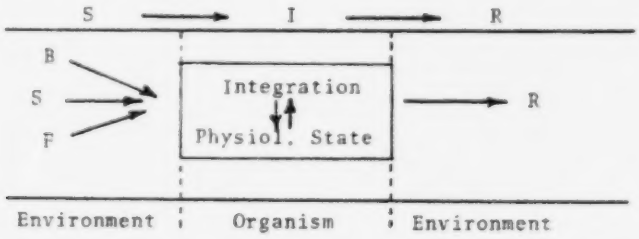
3.1 INTRODUCTION

Over recent years, a great number of sublethal effects of environmental pollutants on aquatic organisms have become known, from all levels of integration, encompassing biochemistry, physiology and behaviour. Some of these findings resulted from tests that show potential for inclusion in a package of tests called a "protocol". The present paper will discuss the suitability of behavioural responses and testing techniques for the development of protocols. I will first try to clarify the rationale for using behavioural responses, their specific position and significance in a "mixed bag" of tests. Second, a number of examples - sometimes taken from ongoing work at the Freshwater Institute, Winnipeg - will illustrate methodological approaches and interpretation of results. Finally, viewpoints and results, problems and promises will be summarized.

3.2 BEHAVIOURAL ASSAYS - WHY?

Of course, one easy answer to this question is: knowledge about behavioural responses to pollutants, or about pollution-induced changes of behaviour, is required if we are to arrive at a comprehensive assessment of toxic effects; behaviour will have to be considered as one of many parameters, e.g. blood chemistry, histology, metabolism, fecundity, etc. However, there appears to be one additional viewpoint setting behavioural reactions apart from other effects.



Environmental entities in form of "factors" (F), "stimuli" (S) or "biota" (B) are impinging on the animal; within the organism, these various environmental conditions lead to specific effects (e.g. change of blood sugar, metabolic level, hormone activity); the resulting behavioural response (R) is based on integration of potentially all underlying physiological events. This generally accepted model has 2 major implications with regard to environmental toxicants:

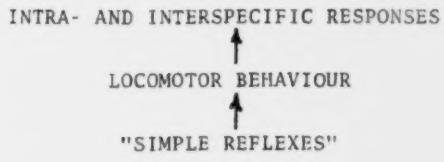
a) Changes or impairments that might have been overlooked or difficult to measure at biochemical or physiological levels can manifest themselves in the behavioural output;

b) Changes or impairments of particular biochemical or physiological functions are often easy to document, but hard to evaluate. More often than not, the question remains: what does it mean to the performance of the whole animal, to its chances for survival, if an enzyme activity is lowered, a sensory threshold increased to some extent? Here, the behavioural output of the animal can be used to assess the relevance or severity of changes of underlying parameters.

3.3 THE STATE OF THE ART

This subheading should not be taken to imply the description and discussion of fully developed standardized assays. They do not yet exist, i.e. very few behavioural (or any sublethal) tests have reached the stage where they are widely applied, following common procedures. However, pertinent literature over the past 5 to 10 years reveals the scope of behavioural responses described in relation to environmental toxicants; some of these responses turn out to be investigated again and again by various authors applying similar techniques, thus indicating potential for standardization. The state of the art then may be described as just reaching a transition to widespread application. Accordingly, this paper will mainly aim at providing an overview, hopefully stimulating further research and development, rather than a review.

I have grouped the various responses described in the literature under 3 headings, implying different levels of complexity. They are not strictly discrete categories without overlaps, but they do indicate different levels of integration that are important with regard to the practicality of assays.



3.3.1 "Simple Reflexes"

In the present context, this somewhat dubious term may serve to indicate a number of responses that appear relatively simple in that they tend to be rather stereotyped, often merely described in a quantal way (they occur or they do not), without as much input from and interactions with endogenous or external variables as responses under the two other headings. For instance, Anderson and Prins (1970) described the use of the propeller tail reflex in rainbow trout. This reaction, a peculiar movement of the tail, is normally elicited by stimulation of the gular region. Anderson and Prins used classical conditioning to establish this response and showed that its display was reduced after sublethal exposure to DDT. Another example is the co-called cough response, apparently a gill clearing mechanism displayed by many fish species exposed to pollutants. Taxic responses, at least their very initial orientational element, could be listed here, too. Disappearance or decrease of positive rheotaxis of fish caged in in-stream or in-plant flow-through tanks has been proposed as an early warning against insufficient water quality (e.g., Besch et al. 1974). Responses as mentioned above and their change or disappearance are easy to document; at the same time, they tend to indicate little in terms of survival value. A response much more complex in sensory, central and motor functions required is maintenance of equilibrium. In fact, this response appears to be so strongly programmed into fish as to become insensitive as an indicator of sublethal impairment: once the fish loses equilibrium it is likely to be close to death. Lindahl and Schwanbom (1971) apparently overcame this problem and turned this response into a more sensitive assay with a graded read-out. They subject their fish to flowing water that is made to rotate in a tube around the direction of flow. The criterion for impairment is a lowering of the "critical r.p.m.", i.e. the maximum number of rotations per minute the fish is able to compensate for.

3.3.2 Locomotor Behaviour

Under this heading we are dealing with a vast number of responses that are more complex in regard to number of underlying functions and external determinants. Locomotor behaviour has many "simple reflexes" as elements or prerequisites (e.g. equilibrium, taxic reactions); beyond that, however, potentially all physiological and environmental conditions may contribute. (In the present context, I will separate biota from environmental conditions; see under 3.3.3, "intra- and interspecific relations"). The advantage of using locomotor responses for sublethal bioassays is a high level of integration, and yet a behavioral output that usually can be objectively described and quantified. Probably, this is why the majority of behavioural tests known at present deal with locomotor behaviour. Because of the wide range of responses and techniques in this area, I will restrict myself to those that appear at present most advanced methodologically, most suitable for standardization and most convincing in regard to assessing deleterious effects of toxicants.

SOME TYPES OF LOCOMOTOR BEHAVIOUR PERTINENT TO ASSAYS

1) Activity
   a) forced
   b) free
2) Drift
3) Spatial Selection ("preference/avoidance")
   a) direct response to toxicant
   b) toxicant-caused response changes

"Forced"-activity tests determine maximum sustained swimming speed, burst speed, scope for activity etc., i.e. criteria for what is called swimming performance. A substantial amount of background data, based on well developed techniques, is available (e.g. Brett 1964, Brett and Glass 1973). Obviously, impairment of swimming performance is significant for survival. Accordingly, a number of authors have used this parameter (MacLeod & Smith 1966, Oseid & Smith 1972, Peterson 1974, Howard 1975).

"Free" locomotor activity refers to species-specific levels and patterns of locomotor activity, characterized by circadian and seasonal rhythmicity. Over the past 10 to 20 years, particularly in European countries, these behavioural patterns have been subject to extensive research (work with fish reviewed by Schwassmann 1971). Several methods for recording these patterns are available, e.g. interruptions of a light beam as a measure for activity as used by Wallace (1975, and this volume). A technique employing ultrasonic beams is presently being developed at the Freshwater Institute. One attractive feature of measuring this response is that it entails continuous 24 hrs-a-day monitoring of the animal. I found that a decrease of activity in whitefish and rainbow trout following sublethal dosages of fenitrothion could be readily documented, as well as subsequent recovery after cessation of exposure to the organophosphate (Scherer, unpublished). Waller & Cairns (1972) and Bengtsson (1974) described alterations of movement patterns caused by exposure to zinc. Still needed, especially for North American fish and invertebrate species, are background data on normal activity patterns and their natural fluctuations. Also, possible relations between free and forced activity remain to be clarified.

Drift, the downstream movement of stream-dwelling invertebrates, has been found to show distinct circadian patterns (Tanaka 1960, Waters 1962). At present it is still a matter of discussion and speculation how these patterns come about, i.e. what makes stream dwelling animals drift as they do under natural, undisturbed conditions. But there is evidence that dirft rates may change significantly when water temperature is elevated (Wojtalik & Waters, 1970) or a pesticide enters a river; Flannagan (1975) observed an incidence of an 85-times increase of Plecoptera drift following mehtoxychlor treatment. It

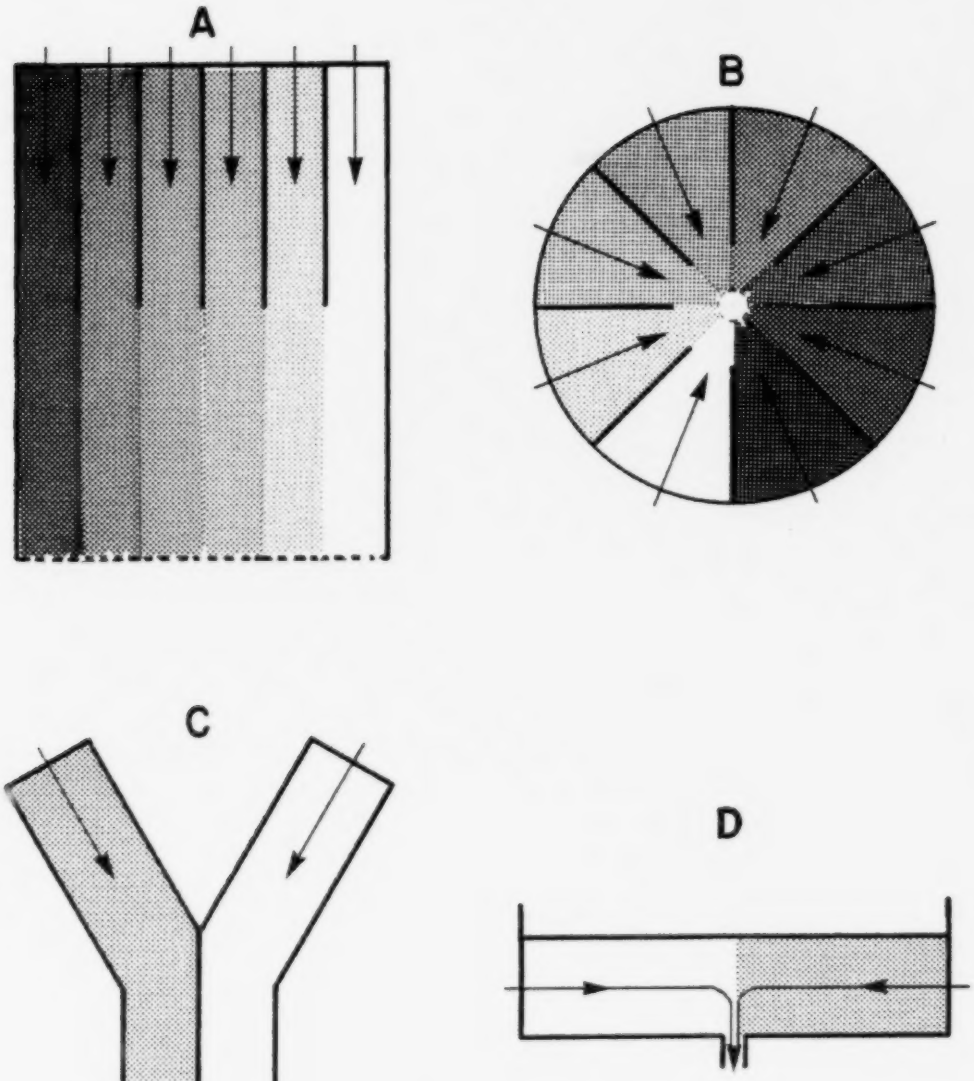


Fig. 1

Basic designs used by various authors to test preference/avoidance reactions of aquatic organisms. Arrows indicate direction of water flow.

A,B: Gradients; e.g. Höglund 1953; Kleerekoper & Mogensen 1963.

C: Y-maze; e.g. Hansen 1969, Folmar 1976.

D: Countercurrent tank; e.g. Sprague 1964, Scherer & Nowak 1973.

appears to me that experiments employing stream channels with well-controlled conditions and close observation of drift behaviour, are necessary to elucidate the drift phenomenon, which may then provide an important assay pertaining to maintenance or abandonment of habitat.

Finally, (cf schematic preceding page) we have to deal with spatial selection by animals. The ability to perceive environmental factors and to select favourable conditions is of utmost importance for survival. From the beginning of their science, ecologists analyzed locomotor responses leading to species-specific distribution patterns within an environment that, be it a forest, a lake, or a stream, is never uniform or homogeneous. With regard to fish and aquatic invertebrates a number of methods and data exist on choice reactions to dishomogeneities or gradients of natural factors such as temperature, light, substratum, food odours, etc. These so-called preference/avoidance responses attracted renewed and increased interest with the advent of pollutants. In experimental design and interpretation of results, however, we should be aware of important differences in testing the reactions to natural gradients of, for instance, temperature or food extract on one, and a toxic pollutant on the other side.

First, we cannot expect a man-made substance to be avoided according to its toxicity; with no history of exposure, no genetically programmed response can be assumed; the animal may even be unable to perceive the toxicant - analagous to the human predicament with regard to carbon monoxide. The rationale for testing the direct response to toxicants then can only be to determine a) whether the species can detect the toxicant at all, and b) if so, whether preference for the toxicant will render it actually more hazardous, or avoidance may provide a chance for escape.

A second point pertains to the testing technique. All basic designs to test choice reactions to natural factors (Fig. 1) have been applied to pollutants as well (e.g. Shelford and Allee 1913, Jones 1947, Höglund 1953, Lindahl and Marcstrom 1958). However, exposing a specimen to a gradient of several toxicant concentrations (A, B in Fig. 1) may alter the response due to toxic action already taking place during the course of test. I therefore opted for a design that provides a reliable separation between pure and toxicant-containing water, with a device for tracing and documenting the specimen's movements into and out of the toxicant accurately (Scherer and Nowak 1973).

The direct response to toxicants in form of preference or avoidance has been reported in numerous papers as indicated above; in fact, at present it appears to become the most popular of behavioural tests. Its relevance is evident provided the two major above mentioned considerations and restrictions are kept in mind. In order to arrive at a measure for sublethal toxic effects, we can investigate the alteration of a known preference /avoidance response to a non-toxic agent (e.g. temperature, light, food) by the toxicant in question (e.g. Bardach 1965, Ogilvie and Anderson 1965, Opuszyński 1971, Scherer et al. 1975), instead of testing the direct response to the toxicant as done most frequently so far. In many cases, with little or no modification the same apparatus can be used for one or the other type of test, and for fish as well as invertebrates (Maciorowski et al. 1977).

3.3.3 Intra- and Interspecific Responses

These are commonly recognized to represent the highest level of integration. Reactions and adjustments to abiotic elements of the environment provide the stage, as it were, for even more complex behaviour patterns leading to pecking orders, territoriality, courting and spawning rituals, predator/prey relations, etc. All these responses can be suspected, and some have been shown to be susceptible to impairment by sublethal levels of toxicants. We need not debate the survival value of an escape response against a predator, or of the

successful completion of a chain of reactions from courting to spawning. In our present context, the problem with these responses is: if at all possible, they are considerably harder to fit into reproducible, standardized laboratory procedures than "simple reflexes" and locomotor behaviour. Probably it is for this reason that no assays close to widespread practical application are available in the area of intra- and interspecific responses.

3.4 SUMMARY AND CONCLUSIONS

Behavioural responses result from integration of underlying biochemical and physiological events; the whole-animal performance can provide crucial information about the significance of effects observed on isolated biochemical or physiological functions. By the same token, the causal mechanisms linking toxicant and response remain a black box as long as the behavioural assay is carried out in isolation.

Behavioural tests are non-destructive; continual, in some cases continuous monitoring is possible - a great advantage for following the development of changes in chronic tests and for analyzing phenomena like acclimation, adaptation, habituation, learning, and recovery from exposure.

Behavioural testing methods often need very little modification to be applicable to various species of invertebrates as well as to fish (e.g. locomotor activity, avoidance testing).

One group of responses or response changes, namely those in the area of intra- and interspecific relations, appear not amenable to standardized testing. It is very difficult, if at all possible, to provide all the biologic and abiotic conditions necessary to arrive at reproducible quantitative results. As a group of reactions proven to be particularly suitable for assays I emphasized locomotor behaviour, including free and forced activity, drift, and preference/avoidance responses.

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3.6 QUESTION PERIOD

V. Zitko, Biological Station, St. Andrews: The solubility of fenitrothion is about 27 mg/l. Why did your results deal with concentrations as high as 100 mg/l?

E. Scherer: You are referring to my tests on the response of goldfish to fenitrothion (Bull. Environ. Contam. Toxicol. 13/4, 1975). There is evidence for several species of fish, and apparently for invertebrates as well (see Maciorowski et al. 1977) that the response to a toxicant is not necessarily monophasic, i.e. a change from avoidance to preference or vice versa may occur when a wide enough range of concentrations is presented; a fact worthy of more investigation. In the case of goldfish and fenitrothion, however, tested from 0.01 to 100 mg/l (the latter target concentration set up with Atlox plus sonication) the response was avoidance only, increasing with concentration over the whole range.

Jerry Payne, Biological Station, St. John's: Behavioural assays are useful if we are trying to pick up some specific response with a sublethal concentration of a toxicant. If we throw in enough of a chemical to make a fish very sick, and probably die a few days after our magic 48 hours, we are probably only picking up an indirect dying response of a sick animal. Then we are no further ahead than in direct toxicity studies.

To throw another complexity into your picture about circadian rhythms, what about cases where toxicants affect levels of certain neurotransmitters?

E. Scherer: I think the first part of your comment underlines that we should stay aware of the difference between sublethal responses and prelethal, irreversible effects. I agree; this probably holds true for whatever parameter you measure, be it behavioural, physiological, histological, biochemical.

As to effects on neurotransmitters: indeed, I found in a joint experiment with Drs. Klaverkamp and Lockhart at the Freshwater Institute that inhibition of AChE caused by an organophosphate pesticide correlated well with a suppression of locomotor activity.

CHEMISTRY IN THE DETERMINATION OF TOXICITY OF CHEMICALS TO AQUATIC FAUNA

VLADO ZITKO*

Zitko, V. 1977. CHEMISTRY IN THE DETERMINATION OF TOXICITY OF CHEMICALS TO AQUATIC FAUNA. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5-AR-77-1, Halifax, Canada. pp. 41-48.

The chemical characterization of water and tested compounds, monitoring of the concentration of tested compounds in water and animals during the tests, identification of breakdown products and metabolites, and structure-activity relationships are discussed. The importance of close cooperation between biology and chemistry is emphasized.

Zitko, V. 1977. CHEMISTRY IN THE DETERMINATION OF TOXICITY OF CHEMICALS TO AQUATIC FAUNA. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5-AR-77-1, Halifax, Canada. pp. 41-48.

La caracterisation chimique de l'eau et de certains composés, l'évolution de la concentration de ces produits dans l'eau et dans les animaux au cours d'éxperiences, l'identification des produits métaboliques et de décomposition et les relations structure-activité constituent l'objet de cet article. On souligne également l'importance d'une coopération entre biologie et chimie.

4.1 INTRODUCTION

The solution of environmental problems requires a close cooperation of professionals trained in various fields. In particular, biology and chemistry are essential in the determination of toxicity of chemicals to aquatic life. A deeper involvement of disciplines, such as biochemistry, physiology, toxicology, and microbiology, is highly desirable.

This paper discusses briefly the role of chemistry in the determination of toxicity of chemicals to aquatic fauna. Chemistry is required not only to measure the concentrations of chemicals in water during the tests. Additional important functions of chemistry include the characterization of water used in the tests, the determination of purity or composition of tested materials, the identification of metabolites and degradation products of tested chemicals, and participation in deriving structure-activity relationships.

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4.2 CHARACTERIZATION OF THE WATER SUPPLY

Water used in toxicity tests is generally well characterized in respect to its inorganic constituents, but not enough attention is given to the organic components. These are important because of their effects on the toxicity of some heavy metals, particularly copper, possible effects on the test animals, and interference in the determination of tested organic compounds.

The measurement of the complexing capacity of water should be included in the routine characterization of the water supply. The cupric ion-selective electrode can be used conveniently for this purpose (Zitko *et al.*, 1973; Chau and Lum-Shue-Chan 1974).

Toxicity testing laboratories generally use a variety of plastic materials in contact with water supplied to the test animals. As a result, the water may contain appreciable amounts of numerous chemicals leached from the plastics. An analysis of compounds eluted by water from commonly used plastic tubings was published recently (Junk *et al.* 1974). Little is known about the toxicity of some of these chemicals and this problem deserves more attention.

Seawater intakes are usually located in the vicinity of wharfs or similar structures treated with creosote. Sea water may thus become contaminated by aromatic hydrocarbons which is frustrating, to say the least, if one is interested in sublethal effects of oil.

4.3 CHARACTERIZATION OF TESTED MATERIALS

The importance of chemical characterization of tested materials cannot be overemphasized. Variable composition of commercial formulations and impurities in compounds under test may cause discrepancies in toxicity evaluations and in comparisons of results between laboratories.

4.3.1 Highly Toxic Impurities

There are a few examples of the presence of highly toxic impurities in certain chemicals. Chlorinated dibenzodioxins may be present in chlorinated phenols and their derivatives, and chlorinated dibenzofurans may occur in PCB's and in chlorinated phenols. Some organophosphate pesticides may contain tetraethyl pyrophosphate (TEPP) or its thioanalogue (sulfo-TEPP).

Impurities such as these may affect the results of the toxicity tests. With improvements in chemical separation and identification techniques and the growing awareness of the importance of impurities, more examples will become available. However, problems of this type are not of major concern to routine toxicity testing. One should, of course, be on the lookout for unexpected toxicological symptoms and try to identify major impurities in tested materials.

The general characterization of technical materials, formulations, and complex effluents is very important in routine toxicity testing and is discussed below.

4.3.2 Pesticides

The first question to be answered in this case is whether the material submitted for testing is the technical pesticide itself ("active ingredient"), or whether it is a formulation. Three basic types of formulations are available: emulsifiable concentrates, wettable powders, and dusts, and each type may be formulated with several concentrations of the active ingredient.

The supplier can usually provide some information on this subject, but the qualitative and quantitative composition of the formulation may not be available readily or is reported only in terms of tradename products, with little information about the chemical

identity and toxicological properties of the components.

The "formulation or active ingredient" question, at times, can be answered simply by visual inspection of the material. The physical form of the active ingredient is given in pesticide handbooks. For example, if a pesticide is crystalline, and the material submitted for testing is a liquid, it is obvious that it is either a formulation or impure active ingredient.

Scanning the infrared spectrum of the material is a simple technique to determine its purity. In addition, the spectrum may provide helpful hints about the nature of the solvent, emulsifier, etc. Reference spectra of many pesticides are available (Gore *et al.*, 1971; EPA 1976). Having established that the material on hand is a formulation, the concentration of the active ingredient must be determined. The supplier may provide this information, but usually only in terms of a tolerance ("not less than . . .") rather than the actual value. The methods of analysis of pesticide formulations are often different from those used in trace analysis. A good source of methods for pesticide formulations is the AOAC handbook (Horwitz 1970).

The World Health Organization provides specifications for pesticides and formulations used in public health programs, and the test methods are described (WHO 1973). It is likely that a similar approach will be adopted eventually for all pesticide formulations.

The solvent and the emulsifier may be separated from the active ingredient by column chromatography, and characterized further. For hydrocarbon solvents, the proportion between aromatics such as benzene, toluene, xylenes, naphthalenes, and aliphatic hydrocarbons should be determined. In the case of the emulsifier, it is important to know whether it is an anionic or a nonionic compound, and in the latter case, whether it is an ether or an ester and what is the respective acid or alcohol. Infrared and ultraviolet spectrophotometry are convenient tools for such a general characterization. Methods to determine the ethylene oxide chain length are also available, but are somewhat more involved (Tobin *et al.*, 1976). A rough estimate of the chain length may be obtained from infrared spectra if several reference compounds are available.

Technical pesticides (active ingredients) also deserve some characterization, particularly if they are liquids rather than crystalline solids. Organophosphates such as fenitrothion, disulfoton, naled, phorate, and the demetons are good examples. Technical products may be quite impure and it is important to record at least the chromatographic profile of the mixture, even if some components remain unidentified. A comparison of such profiles between batches or laboratories could be very useful in explaining differences in the results of toxicity tests.

4.3.3 Oil Dispersants

Before initiating tests with oil dispersants, the solvent and the surfactant should be identified. The solvent may be separated by column chromatography or vacuum distillation and characterized by infrared spectrophotometry. Infrared spectra are again useful in determining the nature of the surfactant. Either the residue after vacuum distillation, or fractions from column chromatography may be used. The common solvents are paraffinic or aromatic oils, and aliphatic alcohols, and the nature of the solvent is important for the assessment of toxicity of the dispersant.

The persistence and toxicity of nonionic surfactants are usually greater for ethers than for esters. Relatively little attention has been given to the formation of hydroperoxides and their decomposition products in oil dispersants during storage, and their toxicological significance is unknown.

4.3.4 Complex Effluents

It is well known and will be mentioned only in passing that the pH and stability of the effluent, indicated by its oxygen demand, deserve the first consideration before the toxicity of these substrates is tested.

A rough fractionation of organic compounds in the effluent into neutral, acidic, and basic fractions may be achieved by solvent extraction. Toxicity tests on these fractions indicate their relative importance in the toxicity of the effluent. The most toxic fractions may be investigated further to attempt a more definitive identification of the constituents. A toxicity balance of the effluent may be eventually constructed to confirm that most of the effluent's toxicity has been accounted for. Good examples of such an approach to the study of complex effluents are available in the literature (Leach and Thakore 1976)

The interest in the composition of the effluent is not purely theoretical. The data may give indications about possible process or "housekeeping" changes, treatment methods, effluent criteria, and compliance monitoring.

4.4 MEASUREMENT OF TOXICANT CONCENTRATION DURING TESTS

4.4.1 Concentration of Toxicant in Water

The need to know the actual rather than the nominal toxicant concentration is generally recognized. Analysis of a larger number of samples in static than in running-water tests and analytical techniques should be as simple and rapid as possible. Existing methods can often be modified to achieve this, since the composition of the test solution is known qualitatively, and interferences by other chemicals can be anticipated. Atomic absorption spectrophotometry for inorganics, ultraviolet spectrophotometry and gas chromatography for organic compounds are usually the most convenient analytical techniques.

For many compounds tested in the mg/l range, atomic absorption and ultraviolet spectrophotometry may be used directly on the water samples. In static tests after about 24 h, the turbidity interferes with optical measurements, and the toxicant must be extracted. Chloroform and diethyl ether are convenient extractants for organics to be measured by ultraviolet spectrophotometry. Hexane or ethyl acetate are suitable for subsequent gas chromatography of organic compounds.

The composition of mixtures such as PCB's, pyrethrum extract, and hydrocarbons, changes during static tests. Such changes always should be noted, even if they may not be appropriately quantified due to unknown detector response factors.

In static tests, the concentration of many compounds decreases exponentially with time, and this observation has been substantiated theoretically (Gillespie *et al.*, 1975). It is not always possible or desirable to perform running-water tests and the toxicity must be determined from static tests and variable toxicant concentrations. The best approach to arrive at an average toxicant concentration is probably the integration of the area under the concentration *vs* time curve.

4.4.2 Concentration of Toxicant in Test Animals

The measurement of toxicant concentration in the test animals always should be attempted to estimate the accumulation coefficient, and to determine the fate of the compound. Such measurements are performed fairly routinely for heavy metals and organo-halogen compounds, but not very often in other cases. The main reasons for this situation are the lack of methods and time. There should be more effort in this direction, not only to provide data for the toxicity tests, but also to gain experience, invaluable in analyses of field samples.

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4.9 QUESTION PERIOD

John Davis: There is some school of thought that the apparent toxicity or bioaccumulation potential of some substances could be analysed or predicted in terms of the partition coefficient in lipoidal material.

Vlado Zitko: The concept of structure-activity relationships is gaining grounds in environmental research. A symposium on this subject was held in 1975. The Proceedings are available (see Zitko 1976).

Peter Hodson: I was very interested in your comments on phthalates in tubing. Have you done enough analyses to allow you to suggest materials or brands?

Vlado Zitko: I don't have sufficient data to suggest materials or brands. The paper given under Gillespie *et al.*, 1975, may give you the general picture.

P. Wells: It would probably be wise to adopt a method of changing some of our laboratory materials more frequently if there were hazards of materials leaching from aged materials.
Peter Hodson: This would maintain a constant high background of phthalates – do we want that?

Ed Pessah: There was a paper 3 to 5 years ago that dealt with growth inhibition. I believe algae were tested against a variety of synthetic materials including a variety of

flexible NC tubings and nylon. I think the only products that were considered acceptable were glass and possibly teflon. These were long-term tests and it is doubtful that we should expect a similar experimental bias potential in our short-term work.

Jerry Payne: I have been trying to use a peristaltic pump in dosing experiments with oil dispersants. Unfortunately both teflon and silicon tubing soften, expand and break. In reference to Ed's comment, a variety of tubing is known to be toxic to various tissue cultures.

Ron Watts: I have not yet found a good list of standard water chemistry parameters that should be recorded for bioassays. Do you advocate such a standard list and if so, would you suggest one?

Vlado Zitko: The list would depend on the type of compounds tested. Among the inorganic parameters it certainly should include hardness, alkalinity, and complexing capacity. The organic components should be at least screened qualitatively and, at this stage, TOC may be the best quantitative measure.

Gordon Craig: You mentioned that using chemical techniques such as partition coefficient evaluation can provide a toxicity balance of compounds in effluents. Has this approach been investigated, was it successful, and how time-consuming was it? Furthermore can the interaction of various constituents in effluents be expressed in toxicity terms using the above techniques? It seems that this approach has a more immediate application in the screening process of new products being marketed for industrial or agricultural purposes.

Vlado Zitko: The application of structure-activity relationship is primarily in the screening process of chemicals.
For complex effluents I would recommend chemical fractionation, combined with toxicity tests on the fractions. The latter determine the relative importance of the individual fractions for the toxicity of the effluent and lead the chemist to the identification of the most toxic components.
Such an approach is used successfully, for example, by B.C. Research on pulpmill effluents.

Hari Samant: We very often analyse for the inorganic components of dilution water but the organic components are largely ignored. I think that rather than trying to standardize dilution water one should place more emphasis on the controls within the test because there are too many variables with respect to the chemical forms in which the materials are found in the water. I think that standardization of water quality without going into the individual speciation of the materials would be a simplistic approach.

THE USE OF AVOIDANCE-PREFERENCE BIOASSAYS WITH AQUATIC INVERTEBRATES

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Maciorowski, H.D., R. McV. Clarke and E. Scherer . 1977. THE USE OF AVOIDANCE-PREFERENCE BIOASSAYS WITH AQUATIC INVERTEBRATES. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5AR-77-1, Halifax, Canada. pp. 49-58.

The potential exposure of aquatic organisms to lethal and sublethal concentrations of toxicants can be assessed by their behaviour in avoidance-preference bioassays. Several avoidance-preference systems have been tested or modified for use with invertebrates. Invertebrate avoidance-preference responses have been monitored for various heavy metals, pesticides and oil. Not all invertebrates are equally suitable for these avoidance-preference tests. Test conditions should be chosen to fit the general biology of the species. The behavioural response may vary with the physiological state of the organism. Although avoidance or preference responses can be detected in avoidance-preference bioassays, the physiological and biochemical mechanisms causing these responses usually are unknown. In agreement with results reported for fish, the direct behavioural response of invertebrates to toxicants is no indication of toxicity. This is illustrated by the avoidance by Gammarus lacustris of 0.15 and 0.46 mg/L Cu^{++} and its attraction to 12.3 and 30.0 mg/L Cu^{++}. The experimental design of avoidance-preference bioassays may be modified to measure the effects of toxicants on other behavioural patterns such as reactions to food stimuli.

Maciorowski, H.D., R. McV. Clarke and E. Scherer . 1977. THE USE OF AVOIDANCE-PREFERENCE BIOASSAYS WITH AQUATIC INVERTEBRATES. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5AR-77-1, Halifax, Canada. pp. 49-58.

La tolérance des organismes aquatiques en présence de concentrations mortelles ou non-mortelles de produits toxiques peut être déterminée ou moyen du comportement ou cours d'essais biologiques d'attirance-répulsion. Plusieurs systèmes d'attirance-répulsion ont été essayés ou ont été modifiés en vue d' utilisation avec des invertébrés. On a surveillé la réaction des invertébrés dans des systèmes d'attirance-répulsion en présence de métaux lourds, de pesticides ou de pétrole. On a constaté que tous les invertébrés ne sont pas également appropriés pour ces essais de tolérance-répulsion. On doit choisir les conditons d'essais qui conviennent le mieux aux conditions biologiques de chacune des espèces. La réaction de comportement peut varier selon l'état physiologique de l'animal. Bien qu'il soit possible de déceler les réactions d'attirance-répulsion au cours de ces essais les mécanismes physiologiques et biochimiques qui causent ces réactions demeurent pour la plupart inconnus. La réaction directe de comportement des invertébrés vis à vis les produits toxiques, tout comme c'est le cas pour les poissons, n'est pas un indice de leur toxicité. Le Gammarus lacustris montre bien cette particularité; il manifeste une réaction de

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répulsion aux concentrations de 0.15 et 0.46 mg/L Cu^{++}, mais se laisse attirer aux concentrations de 12.3 et de 30.0 mg/L Cu^{++}. Le devis expérimental des essais biologiques d'attirance-répulsion peut être modifié pour mesurer les effets de substances toxiques sur d'autres manifestations du comportement habituel, telle la réaction au stimulus de la nourriture.

5.1 INTRODUCTION

Behaviour affects the survival of aquatic invertebrates and reflects the integration of many biochemical and physiological processes. Therefore behaviour is an important area to examine when investigating the effects of toxicants on aquatic invertebrates.

Many behaviour patterns of aquatic invertebrates, such as dispersal of young, movement to pupate, drift, feeding and reproductive behaviour, may be affected by exposure to toxicants but not all of these behaviour patterns are suitable for monitoring in the laboratory. Among the behaviour patterns of aquatic invertebrates which have been monitored in the laboratory are activity (Rees 1972; Wallace et al. 1975; Wildish 1970; Williams 1969), drift (Fahmy and Lush 1975), rheotaxis (Vobis 1973) and avoidance-preference (Costa 1966; Hansen et al. 1973; Maciorowski and Benfield 1976; McLeese 1973, 1974, 1975; Percy 1976). Avoidance-preference tests are useful in assessing effects of toxicants on aquatic invertebrates since they provide information on the potential exposure of aquatic invertebrates to lethal and sublethal concentrations of toxicants.

This paper provides an overview of the use of aquatic invertebrates in avoidance-preference tests by discussing avoidance-preference test systems, the selection of aquatic invertebrates for use in avoidance-preference tests, results of some avoidance-preference tests with the amphipod Gammarus lacustris, and the use of modified avoidance-preference tests with simultaneous presentation of a toxicant and a second non-toxic stimulus.

5.2 AVOIDANCE-PREFERENCE TEST SYSTEMS

Avoidance-preference tests monitor the ability of the test organism to detect the presence of a toxicant and to respond to the toxicant by moving into the toxicant or into pure water. Time spent in the two media is recorded and the movement of the test organism during the experiment may be traced (Costa 1966), monitored through a scope eye-piece electronically wired to two time clocks and an automatic strip chart recorder (Scherer and Nowak 1973) or monitored with a portable television camera and receiver (Westlake and Lubinski 1976).

Avoidance-preference tests with aquatic invertebrates have used various test tank designs. Maciorowski and Benfield (1976) exposed crayfish, Cambarus acuminatus, to cadmium in the open-field system (Westlake and Lubinski, 1976) in which the toxicant and water flow side by side in the same direction across a 50 x 50 cm observation area (Fig. 1).

Hansen et al. (1973) exposed grass shrimp, Palaemonetes pugio, to pesticides in the double Y maze (Hansen et al. 1972) in which a toxicant is introduced into one Y shaped arm and pure water into the other and the two media drain into the circular holding area (Fig. 2). Disadvantages of the double Y maze are that aquatic invertebrates may be affected by eddies and areas of no current in the circular holding area and toxicant concentrations in the circular holding area may not be homogeneous because of the turbulence.

Costa (1966) exposed Gammarus pulex to several toxicants in the counter-current tube (Jones 1947) in which a toxicant is introduced into one end of the tube and pure water into the other end (Fig. 3). The two media meet at the center of the tube, draining there and create a toxicant-water interface. The rectangular counter-current system (Scherer and

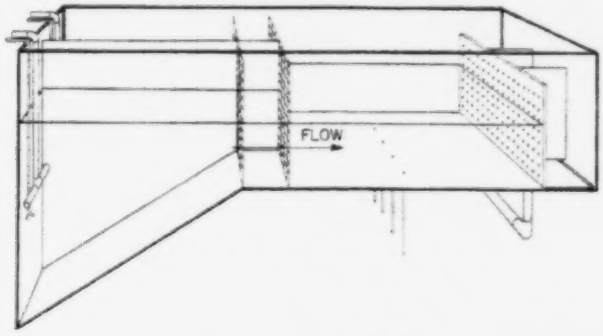

Fig. 1. Diagram of the open-field system (Westlake and Lubinski 1976).

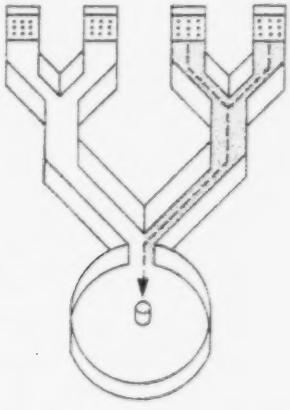

Fig. 2. Diagram of the double Y maze (Hansen et al. 1972).

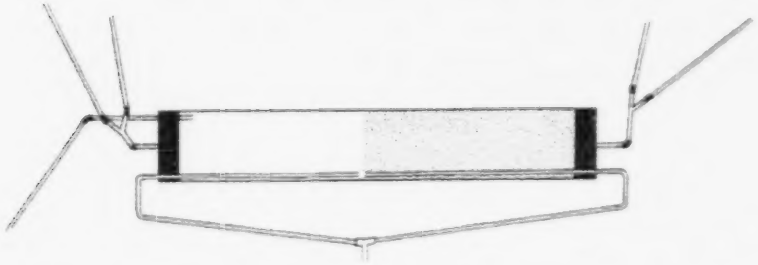

Fig. 3. Diagram of the counter current tube (Jones 1947).

Fig. 4. Diagram of the rectangular counter current system (Scherer and Nowak 1973).

Nowak 1973) is based on the same principle as the counter-current tube (Jones 1947) but differs in using a rectangular tank as the main component of the apparatus instead of a tube. We reduced the size of the rectangular counter-current system (Scherer and Nowak 1973) for use with G. lacustris (Fig. 4). One possible disadvantage of both counter-current systems is that the change in current direction at the drain may affect the behaviour of aquatic invertebrates by, for example, reducing the frequency of interface crossings.

We chose the reduced rectangular counter-current system (Scherer and Nowak 1973) because we found it easier to maintain a toxicant-water separation in this chamber than in the open-field tank (Westlake and Lubinski 1976) and the double Y maze described by Hansen et al. 1972 (see also Scherer, this volume). In addition construction of this system, excluding the tracking device, was easy and inexpensive.

5.3 THE SELECTION OF AQUATIC INVERTEBRATES FOR USE IN AVOIDANCE-PREFERENCE TESTS

APHA et al. (1975) provide general criteria for selecting test organisms for use in toxicity tests but not all aquatic invertebrates meeting those criteria are suitable for use in avoidance-preference tests since avoidance-preference tests have certain additional requirements.

Motility is a major criterion in selecting aquatic invertebrates for use in avoidance-preference tests. Normal swimming and crawling speed and stamina of test organisms should enable them to move freely throughout the avoidance-preference apparatus without interference from the currents or the drain. Therefore more sedentary organisms and tube dwellers are less suitable for use in avoidance-preference tests. Most avoidance-preference test systems require the use of test species, such as Gammarus, which mainly move horizontally, rather than test species, such as Daphnia, which mainly move vertically. Also the rate of movement should be considered since extremely rapid or slow moving species are more difficult to monitor.

Other behavioural characteristics of aquatic invertebrates affect their suitability for use in avoidance-preference tests. Test organisms should not exhibit rheotactic behaviour, which is the orientation of an animal to a water current (Pardi and Papi 1971), at flow velocities occurring in the avoidance-preference system since rheotactic behaviour would restrict the free movement of the test organism. Territorial species may select an area of the test apparatus as a home range and remain inactive. Thigmokinesis is a response to contact and high intensity of contact stimulation usually results in low activity. Hence species exhibiting positive thigmokinetic behaviour would not swim freely throughout the apparatus.

Activity of aquatic invertebrates is affected by current, light, substrate, food, time of day and time of year (e.g. Wallace et al. 1975). Therefore these factors, and any others such as health, reproductive status and temperature which may affect behaviour, should be taken into account when designing avoidance-preference experiments and interpreting the results.

We considered G. lacustris to be a suitable test organism since it is sensitive to changes in the environment and definite signs of stress can be easily observed (Gaufin et al. 1965), it moves mainly horizontally, and it was not apparently hindered in its movements by the turbulence at the toxicant-water interface or by the current velocity (0.3 cm/s) required to maintain a toxicant-water interface in the rectangular counter-current system.

5.4 RESULTS OF SOME AVOIDANCE-PREFERENCE TESTS WITH GAMMARUS LACUSTRIS

We exposed Gammarus lacustris to eight copper (added as copper sulphate) concentra-

tions; five amphipods were tested individually at each concentration. Each individual was allowed to habituate to the test apparatus for five minutes; then time spent in each half of the tank was recorded first during a ten minute trial in which water was introduced at both ends, and secondly during a ten minute trial in which copper sulphate was added to one end and water to the other end of the chamber. The arcsin transformation (Snedecor and Cochran, 1967) was applied to the percent time data. The Student's t-test determined if there was a statistically significant avoidance of or preference for each copper concentration by comparing the percent time spent in the copper during copper-water trials with 50%, the percent time at which there is neither avoidance nor preference. For tests at each copper concentra- the paired t-test determined if there was a statistically significant change, from the water-water trials to the copper-water trials, in the percent time spent on the side receiving the copper. There was no significant bias for one side during the water-water trials for each concentration. Full details of our experiments will be published elsewhere.

Gammarus lacustris showed a statistically significant avoidance of 0.19 and 0.46 mg/L Cu^{++}, a statistically significant preference for 12.3 and 30.0 mg/L Cu^{++} and no statistically significant avoidance or preference at 0.05, 0.93, and 1.39 and 4.2 mg/L Cu^{++} (Table 1). The percent time spent on the side receiving the copper (Table 1) changed significantly ($p<0.05$) from the water-water trials to the copper-water trials for those copper concentrations at which there was a statistically significant avoidance or preference and also for 4.2 mg/L Cu^{++} at which the preference was not statistically significant. The results of similar experiments with sodium sulphate at concentrations of sulphate the same as in the copper experiments showed no significant avoidance or preference by G. lacustris, indicating that the copper ion was the cause of the behavioural responses in the copper experiments. We found that the 96-hr LC50 of G. lacustris was 0.06 to 0.32 mg/L Cu^{++} when exposed to copper sulphate in static bioassays.

Table 1. Results of avoidance-preference tests with Gammarus lacustris exposed to copper sulphate.

Copper Concentration (mg/L Cu^{++})	Average % Time Spent (n=5) in Side of Tank to Which Copper Was Introduced		Statistical Significance of Avoidance or Preference	
	Water-Water Trials	Copper-Water Trials	Calculated t	Significance ($p=0.05, t_4=2.78$ $p=0.01, t_4=4.60$)
0.05	54.4	56.2	0.67	$p>0.05$; no significant response
0.19	65.5	33.1	-5.29	$p<0.01$; avoidance
0.46	61.7	33.6	-4.64	$p<0.01$; avoidance
0.93	44.0	54.4	1.03	$p>0.05$; no significant response
1.39	62.3	48.1	-0.53	$p>0.05$; no significant response
4.20	40.2	68.6	2.65	$p>0.05$; no significant response
12.30	59.3	78.8	9.20	$p<0.01$; preference
30.00	44.1	78.3	7.25	$p<0.01$; preference

Our avoidance-preference results contrast with those of Costa (1966) who observed that G. pulex avoided concentrations of 0.32, 31.8 and 318.0 mg/L Cu^{++} (Table 2) but the behavioural differences could be due to interspecific variation or differences in water quality and experimental techniques. However, our results are somewhat similar to those of Hara and Scherer (in prep.) with whitefish, Coregonus clupeaformis, which preferred 1.0 to 30.0 mg/L Cu^{++} and avoided 0.01 to 0.50 mg/L Cu^{++}, and those of Jones (1947) with sticklebacks, Pungitius pungitius, which preferred 31.8 and 318.0 mg/L Cu^{++} (Table 2).

Table 2. The avoidance-preference responses of some aquatic invertebrates and fish exposed to copper sulphate solutions.

Approximate Copper Concentration (mg/L Cu^{++})	Gammarus pulex (Costa 1966)	Gammarus lacustrus (this paper)	Coregonus clupeaformis (Hara & Scherer in prep)	Pungitius pungitius (Jones 1947)
0.05	not tested	no response	avoidance	not tested
0.50	avoidance	avoidance	no response	not tested
1.00	not tested	no response	preference	not tested
5.00	not tested	no response	preference	not tested
30.00	avoidance	preference	preference	preference
300.00	avoidance	not tested	not tested	preference

Aquatic invertebrates may avoid and prefer lethal concentrations of toxicants as do fish. We found that G. lacustris avoided copper concentrations 1 to 2 times greater than the 96-hr LC50 but preferred copper concentrations 40 to 300 times greater than the 96-hr LC50. Rainbow trout, Salmo gairdneri, avoided some lethal concentrations of chlorine but preferred an intermediate concentration which was also lethal (Sprague and Drury 1969), and sticklebacks, Gasterosteus aculeatus, avoided low concentrations of lead nitrate but preferred high concentrations (Jones 1947).

Causes for the avoidance or preference reaction to potentially lethal concentrations of toxicants are not understood. Jones (1947) attributed the preference of sticklebacks to high concentrations of copper sulphate to "stupefaction" in the copper sulphate solution. We did not observe any evidence of "stupefaction" in our experiments with Gammarus lacustris and copper.

The change in avoidance-preference behaviour at different toxicant concentrations may result from changes in the sensitivity of chemoreceptors since Hara and Scherer (in prep) observed that 0.25 mg/L Cu^{++}, which was avoided by whitefish, depressed their olfactory sensitivity whereas 2.54 mg/L Cu^{++}, which was preferred by whitefish, enhanced their olfactory sensitivity. We are unaware of data linking avoidance-preference reactions of aquatic invertebrates to chemosensory processes. Atema and Stein (1974) speculated that crude oil interfered with chemoreception when the oil affected the feeding behaviour of the lobster, Homarus americanus; however, histological examination showed no morphological change in the chemoreceptors of the lobsters.

5.5 MODIFIED AVOIDANCE-PREFERENCE TESTS WITH SIMULTANEOUS PRESENTATION OF A TOXICANT AND A SECOND NON-TOXIC STIMULUS

Avoidance-preference tests may be used to determine the change in response of aquatic invertebrates to a non-toxic stimulus during simultaneous exposure to a toxicant as has been done with fish. The non-toxic stimulus may be chemical, such as a host odour for symbiotic or parasitic species, a food odour or a pheromone, or physical, such as temperature (Ogilvie and Anderson 1965) or light (Scherer 1971, 1976). For instance a food odour still attracted lobsters, Homarus americanus, when low concentrations of fenitrothion (McLeese 1974) or kraft mill effluent (McLeese 1973) were present. However, the feeding behaviour of lobsters was depressed during simultaneous presentation of food extract and copper concentrations 4 to 80 times greater than the lethal threshold for copper whereas the feeding behaviour was not affected by phosphamidon concentrations 25 to 1000 times greater than the lethal threshold for phosphamidon (McLeese 1975). Such results suggest that the effects of toxicants on functions connected to feeding are independent of the functions leading to lethality.

5.6 CONCLUSIONS

Avoidance-preference tests provide information on the ability of aquatic invertebrates to detect and avoid lethal and sublethal concentrations of toxicants. Therefore these tests should be included in a package of tests called a protocol.

The results of avoidance-preference tests show that aquatic invertebrates may both avoid and prefer lethal concentrations of a toxicant depending upon the specific concentration. Although behavioural responses can be detected in avoidance-preference tests, the physiological and biochemical mechanisms causing these responses usually are unknown.

Avoidance-preference tests may be modified to determine the effect of a toxicant on a second non-toxic stimulus such as a food odour or a pheromone.

5.7 ACKNOWLEDGEMENTS

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5.9 QUESTION PERIOD

Jerry Payne: How long when you are exposing your animals do you keep them in the container? I worry about habituation or adaptation.

Heather Maciorowski: The amphipods were kept in the chamber for a 5 minute acclimation period, a 10 minute water-water trial and a 10 minute toxicant-water trial. In preliminary experiments there was no statistically significant difference in recorded response in the chamber during the water-water trials following 5 minute, 15 minute or 30 minute acclimation period. From the results I felt there that behaviour would not change due to habituation during the trial with each Gammarus lacustris.

Gordon Craig: The behavioural methodologies presented today appear to suffer from one common limitation. The experimenter must test one organism at a time. Peter Hodson said that replication is necessary to define the variation of the response. This means that one person must sit with one organism a number of times. This seems rather time consuming. Is it not possible to make use of photo recording instruments (camera, video) and expose more organisms (20) simultaneously? What are the limitations of this approach?

John Sprague: I once did avoidance experiments by testing 10 fish at the same time. Comparison showed that it was much more powerful to use one fish at a time and repeat it ten times. For one thing you can more easily keep track of one fish; for another the fish tend to school giving a statistical bias to the trial.

Heather Maciorowski: It is possible to make use of automatic recording instruments. I believe the system described by Westlake and Lubinski (1976) has been modified to include photocells which sense and transmit information on the movement of test organisms to a computer.

Peter Doudoroff: Are the results you got related to time spent in the polluted environment? If so, I wonder whether you have an apparent preference if every time the animal gets into the polluted environment its movements slow down.

Heather Maciorowski: The results are related to the percentage of the 10 minute water-toxicant test period the Gammarus spent in the copper and also the change in response between the water-water trial and the water-toxicant trial.

The amphipods did not appear to slow down when entering the copper. Instead, when the amphipods entered the pure water they swam "erratically" and quickly re-entered the copper side at which time "normal" swimming activity resumed.

Ron Wallace: Heather, I want to make a few points about your presentation. First, I am at a disadvantage as you did not state your materials or methods in your paper, so discussion of results is difficult. From a cursory perusal of your data I think it would be possible to postulate quite different conclusions than those presented. However, aside from that, your comment about your gammarid being "left handed" really bothers me. If your apparatus tended to collect, in control runs, more animals on the left side, then that indicates a serious experimental deficiency, one that would essentially mask any experimental results. Again, as I will stress in my paper to follow, it is vital to understand your animal and its behaviour very thoroughly before using behaviour as a test.

Also, in your presentation you draw comparisons between several different invertebrate species. I would point out that this is highly dangerous, even at the best of times, because of interspecific differences.

I apologize for the lengthy comments, however, as I follow you with my paper you can have plenty of opportunity to form a rebuttal. I shall stress most of my concerns about

your experimental designs in the course of my paper.

Heather Maciorowski: Bias for one side of the chamber during the water-water trial is a concern, but the paired t-test showed statistically significant changes in amphipod behaviour when various copper concentrations were introduced suggesting that behaviour changes occur irrespective of any bias.

In reply to your second question, I did mention during my presentation that variation in response may occur between species and the summary table illustrates this. One purpose of my table was to show the interesting parallel between results obtained for white-fish (Hara and Scherer, in prep) and our own findings.

A DISCUSSION ON THE USE OF BEHAVIOUR BY GAMMARUS PSEUDOLIMNAEUS BOUSFIELD IN EVALUATING ENVIRONMENTAL STRESS

RONALD R. WALLACE*

Wallace, R.R. 1977. A DISCUSSION ON THE USE OF BEHAVIOUR BY GAMMARUS PSEUDOLIMNAEUS BOUSFIELD IN EVALUATING ENVIRONMENTAL STRESS. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5AR-77-1, Halifax, Canada, pp. 59-67.

An apparatus was constructed to automatically monitor the activity of G. pseudolimnaeus in the laboratory. Variables such as current speed, light, food and pollutants were introduced into carefully controlled situations and the change of behavioural activity was noted.

Although G. pseudolimnaeus has been studied extensively in the field, our results indicate that a considerable amount of new data may be acquired by an experimental, laboratory approach to behavioural studies of aquatic invertebrates.

Wallace, R.R. 1977. A DISCUSSION ON THE USE OF BEHAVIOUR BY GAMMARUS PSEUDOLIMNAEUS BOUSFIELD IN EVALUATING ENVIRONMENTAL STRESS. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5AR-77-1, Halifax, Canada, pp. 59-67.

Un appareil construit pour cette experience a servi à contrôler automatiquement l'activité du G. pseudolimnaeus en laboratoire. Certaines variables, telles la vitesse du courant, l'éclairement, la nourriture et les polluants, introduites dans des situations minutieusement contrôlées, ont amené des changements de comportement qui furent notés avec soin.

Le G. pseudolimnaeus a fait l'objet d'études exhaustives dans son milieu, mais les résultats obtenus en laboratoire indiquent que de telles expériences, appliquées au comportement des invertébrés aquatiques, peuvent apporter un nombre appréciable de nouvelles données.

6.1 INTRODUCTION

I want to make two general points to you today, those being:

(1) It is time we spent more effort in exploring the possibilities of using behaviour of invertebrates for the testing of toxicants.

(2) While some aquatic invertebrates have been recognized as being somewhat easier to maintain and use in toxicological tests than fishes, we must recognize that the behaviour of these animals is not necessarily simple and, indeed, may be rather more complex than field tests have indicated.

Much of the data which I will cite, has already appeared in print (Wallace et al., 1975) however, I will also present data which were not included in that earlier paper.

On my first point noted above, I believe that there is a growing recognition of the value and facility with which aquatic invertebrates may be used in the testing of toxicants. More and more experiments are being carried out using invertebrate behaviour in tests. However, I feel I must now sound a warning or at least a note of caution, and that brings me to my second point.

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My earlier work done on G. pseudolimnaeus was attempted because the behaviour of this, and other closely associated species, had been studied extensively by field workers. Indeed, there was an extensive literature which, I hoped at the time, would serve as the basis for manipulative/experimental approaches to toxicological problems in the laboratory.

However, as you will see, once laboratory studies were begun (enabling far more carefully controlled conditions, and much more precise observations) we discovered that many conclusions on the behaviour of this species, which were based principally on field observations, were questionable, if not valid. I will state my warning to you more precisely: Unless careful and thorough laboratory studies are done on the behaviour of aquatic invertebrate species, serious errors in interpretation of toxicological data may be made. In short, know your test species, especially if behaviour is to be used as a criterion in experiments. The temptation to rush into behavioural testing of toxicants with aquatic invertebrate species must be avoided, until such time as an adequate knowledge of the factors influencing their behaviour has been obtained. As you will see, our early laboratory attempts to validate and standardize assumptions about the behaviour of a species which had been extensively studied in the field, forced us to recognize that the behaviour of this invertebrate was much more complex than was widely recognized at the time. Considerable testing and elaboration of behavioural traits was necessary before any toxicological evaluations could be attempted.

6.2 MATERIALS AND METHODS

The experiments were done in two fibreglass tanks, each of which was an oval channel which held 11 litres of water. A paddle-wheel, driven by an electric motor, caused the water to flow in a clockwise direction. A hole in the bottom of the channel on the side opposite the paddle-wheel was covered with clear Perspex, the edges of which were bevelled to minimize water turbulence, and plastic baffles formed a narrow passage 3 cm wide by 40 cm long in mid-channel over the Perspex (Fig. 1). When the motor was running, the current in mid-channel ranged from 3 to 5 cm/sec and that through the narrow passage was 24 cm/sec.

The bottom of the tank was covered with two layers of glass marbles 15 mm in diameter, except for the narrow passage which was always kept clear of obstructions. A cylindrical metal box held a light directly above the narrow passage, and below it were two copper tubes (13 mm [I.D.] wide, 12 cm long and 2 cm apart) which were attached vertically above the Perspex window (Fig. 1).

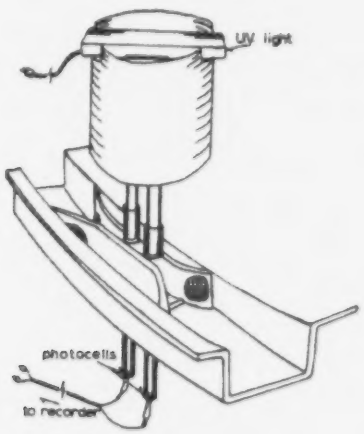

Fig. 1. A cutaway drawing of a section of the tank showing baffles, photocells and the tubes for directing beams of light through the channel.

Two similar copper tubes were fastened beneath the Perspex directly in line with the upper tubes (Fig. 1) and each held a photocell (Clairex Photoconductive cells type CL5M4), which was placed at the bottom to provide maximal shading from extraneous light. The photocells on each tank were connected through an amplifier to one pen on a Honeywell twinpen chart recorder (Electronik Model 194), which was operated at slow speed with two ink colours. The light in the metal container could shine only down the copper tubes and on to the photocells, so any specimens moving through the passage caused an interruption that was recorded.

Dusk and dawn were simulated by an automatic timer switch which dimmed or brightened the lights during the nocturnal phase, except for the U.V. detector light. Light cycles were adjusted so that they were the same as those found in the field at the times of collection of specimens.

Specimens of G. pseudolimnaeus were collected from the Credit River, Ontario with hand nets, and were held in the cold room in aerated pails of water.

The Gammarus were fed with an aquatic fungus (Humicula grisea Traaen) which is known to be a preferred food of G. pseudolimnaeus (F. Barlocker, personal communications).

6.3 RESULTS

Earlier field work indicated that G. pseudolimnaeus exhibited a distinct diurnal pattern of activity, a pattern which was strongly influenced by light intensity and also by seasonal factors (Holt and Waters, 1967; Chaston, 1972; Waters, 1972). Such a pattern was found in our initial laboratory experiments (Fig. 2), however, more subtle complex factors than light cycles were apparently influencing our results.

Initially, several recordings of the activity of specimens were begun shortly after the Gammarus was captured and it was found that the nocturnal activity of the specimens became less each night after capture (Fig. 2).

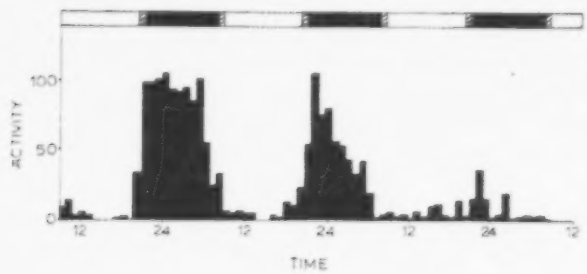

Fig. 2. The activity of Gammarus shortly after capture. 75 specimens collected in August. Water temperature 15°C, current running and food present. Ordinate: number of specimens counted/hour.

Also, the type of light used in the detector beam influenced the activity of the Gammarus near the photocell detectors (Fig. 3). I.R. illumination was apparently detected by the animals and inhibited their movements. When, however, the U.V. light source for the detector was substituted, the classical pattern of night activity was observed.

We also confirmed earlier reports noting that diurnal activity for this species was more pronounced during summer months (Fig. 4), especially when there was no current present (Fig. 5 and 6). However, the mean levels of activity were also greatly influenced by the presence or absence of current in this species which is found normally in running waters. On many occasions, a bigeminus pattern of nocturnal activity was observed both with and without current. Selected examples are depicted in Fig. 5 and 6. None are shown for the winter as no clear nocturnal pattern occurred during that season. The results indicate in general that nocturnal peaks of activity were most numerous during the summer, especially in still waters.

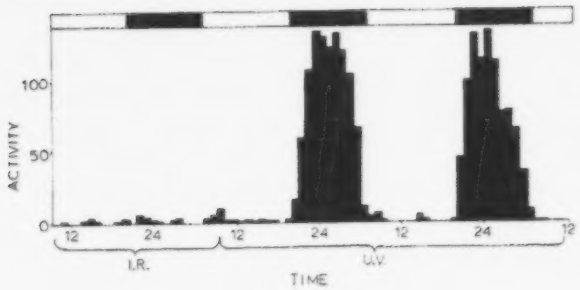

Fig. 3. The activity of Gammarus as influenced by U.V. and I.R. detector lights. Conditions as in Fig. 2

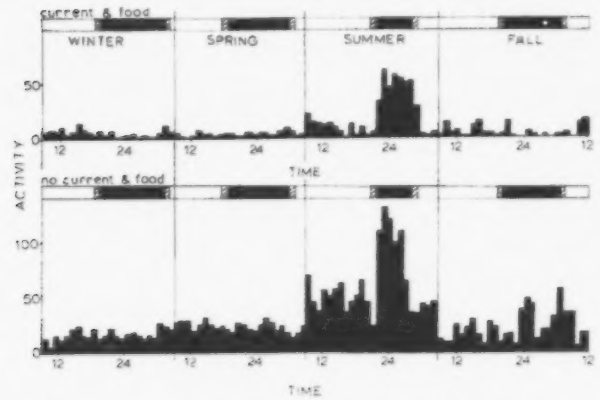

Fig. 4. A comparison of the seasonal activity of Gammarus. Ordinate: mean numbers counted/hour. Upper: current running and food. Lower: no current and food.

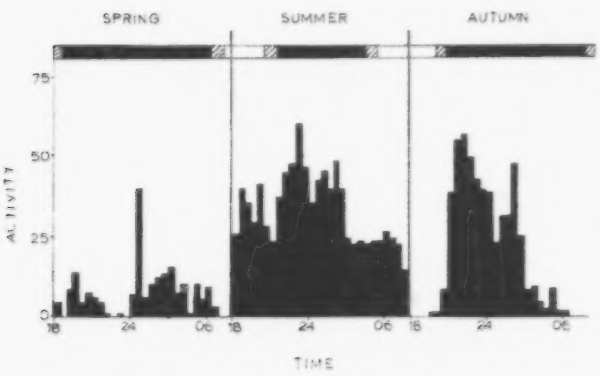

Fig. 5. Patterns of activity of Gammarus during darkness. Ordinate: mean numbers counted/hour. Food present. Current running.

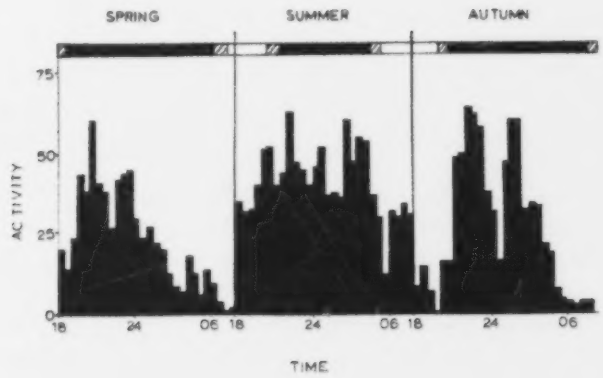

Fig. 6. As per 4, except current stopped.

Changes in photoperiod also produced unexpected results. Specimens were acclimated for several days at the photoperiod that occurs naturally at that season. Then two tests were done, each lasting for 4 days in the presence of food. When the water was flowing, the lights were left on for 24 h, and the normal photoperiod was resumed at 09:00 hours the next day. Then the current was stopped, the lights were left on for 24 h and the normal photoperiod was resumed at 09:00 hours the next day. The test was then repeated.

When the water was flowing, the activity during the 24 h period of light was less than that during the second 24 h period when the normal photoperiod had been resumed, and a bigeminus pattern of activity was observed on this second night (Fig. 7). When there was no flow, the activity increased dramatically, and during the 24 h of continuous light, a distinct bigeminus pattern occurred at almost precisely the time when it would have been dark during the normal photoperiod (Fig. 7). Maximum values of activity declined with time and a less marked nocturnal bigeminus pattern was observed on day 4, when the normal photoperiod had been restored.

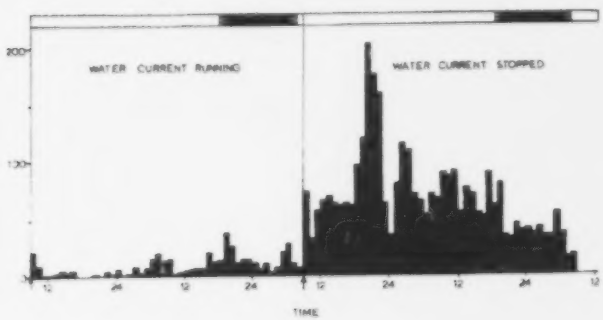

Fig. 7. The effect of altered light cycles on the activity of Gammarus. Temperature 15°C and food present. (a) Current running. (b) Current stopped. Arrows indicate times when the current was started or stopped. Ordinate: numbers of specimens counted/hour.

Substrate/current interactions were also seen to exert an influence upon behaviour. Specimens were placed in a tank with the usual substrate of glass marbles, and recordings were made while the water flowed for 24 h and was then still for 24 h. The procedure was then repeated without the substrate and this set of observations which had lasted 4 days, was then repeated. Food was always present in the tanks and the photoperiod was the same as that occurring naturally.

During the light period with the water flowing, the presence of substrate reduced the activity somewhat, but during the dark period, it made little difference. When the water was still, the absence of substrate apparently increased and depressed activity during the light and dark periods respectively (Table I).

Table I. Mean numbers of specimens (two replications) passing over photocells per 24 h. 225 specimens per tank, water temperature 15°C and food present.

Conditions	Total activity during daylight	Total activity at night (including twilight)
Current running:		
Substrate present	409	230
No Substrate present	505	210
Current stopped:		
Substrate present	516	536
No Substrate present	612	416

Tests with toxicants were attempted only at such time when our experiments allowed us to make accurate predictions as to the various influences which the several factors such as current, light regime, season, substrate and food had on behavioural activity (a process which required several years).

For our preliminary tests, salt was added to a test tank and the mean activity per day was compared with that of a tank which was uncontaminated (Fig. 8). We noted that activity in the tank contaminated with salt, rose markedly above that in control tank, and rapidly accelerated after the concentration rose above approximately 1500 mg/l chloride.

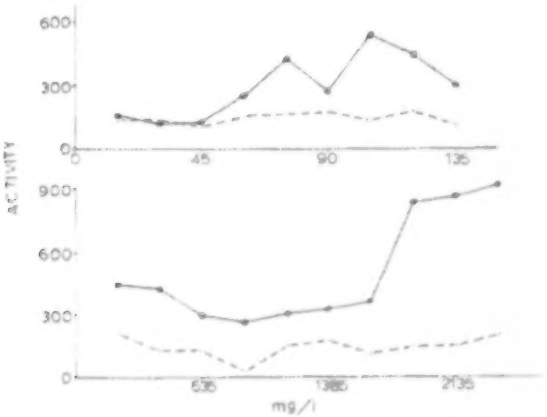

Fig. 8. The effect of increasing NaCl concentrations on the activity of Gammarus. Ordinate: mean numbers counted/day-night (the NaCl concentration was increased daily). Abcissa: mg/l chloride.

6.4 DISCUSSION

The Gammarus both drifted passively and swam actively over the photocells when the current was running, but the tanks must be regarded more as activity chambers than as devices to measure drift, especially when there was no current.

6.4.1 The Influence of Current on Activity

Although there was a seasonal variation on total activity, recorded levels of both diurnal and nocturnal movement were higher when there was no current than when it was running.

Our experiments show that there was a great increase of the activity in G. pseudolimnaeus when the current was turned off.

In our experiments, the gammarids were usually most active after the starting or stopping of the current, and they remained active during the first 24 h of experiments, lasting 3 or 4 days. Hughes (1970) also found a general decline in the rate of drift of G. pulex after several days in conditions of constant flow, and it was because of this phenomenon, that we acclimated all our specimens before using them in experiments. These findings strongly suggest that gammarids initially undertake active exploration of new or changing habitats for about 24 h, and only after this do they become less active.

6.4.2 The Influence of Food on Activity

Although data are not presented here, we found (Wallace et al., 1975) that when food was absent, very marked activity occurred just after the current was started and this was presumably the passive drift for short distances, followed by the move upstream towards food. Large areas of substrate could be quickly searched for food in this way while, at the same time, the short upstream movements could serve to maintain populations and complete a possible 'feeding cycle'.

In the experiments without food, when food was eventually added, only slight decreases in the levels of activity were found. This is puzzling, as many specimens came to the food soon after it was placed in the tank, and Hughes (1970), found that the activity of G. pulex was greatly reduced after introduction of food to his current tanks. In our experiments, it is possible that not enough was added to reduce the activity for long periods, but it is also possible that movement of gammarids seeking the new food source compensated for the reduction in activity of specimens which were eating.

6.4.3 The Influence of Light on Activity

Holt & Waters (1967), found in field experiments that the drift of G. pseudolimnaeus was exogenously controlled, and that "if an endogenous rhythm exists at all, it is very weak and is influenced strongly by environmental light conditions". It is therefore surprising the diel periodicity was not more pronounced in our experiments. Periodicity was hard to detect in specimens collected during summer, but activity during the day was also comparatively high. It should be recalled that the activity recorded by the photocells, was not necessarily "drift", but may be more indicative of the basic levels of activity which may cause the drift in streams.

The most pronounced nocturnal activity occurred during the first 24 h after the current was started or stopped, especially during the summer. This suggests that complex interactions between light current and food, affect the activity of G. pseudolimnaeus.

In our experiments, when there was no current and the lights were on continuously for 24 h, peak levels of activity (with a distinct bigeminus pattern) were reached at precisely the times when the lights normally would have been off (Fig. 7). This certainly suggests an endogenous component in the pattern.

Comparisons of data obtained throughout the year indicated two major rhythms of activity during the summer. Muller et al. (1970) found a similar seasonal pattern of activity for G. pulex, and Chaston (1972) cited evidence which suggested that "seasonal variations in drift levels were related to changes in the propensity of benthic animals to enter drift in relation to stages in their life cycles". Lehmann (1967) found that the seasonal change in the rate of drift of G. pulex was influenced by factors such as sex-finding behaviour of the males during the breeding season, temperature and population density. In our study, the higher activity of G. pseudolimnaeus is probably also related to a cycle of breeding. Hynes & Harper (1972)

found that G. pseudolimnaeus begins to breed in the Credit River in February and continues through July.

The drift of some aquatic invertebrates thus apparently results from exogenous and endogenous components in the nocturnal pattern of activity. Factors such as the onset of darkness, cause increases in activity which result in drift. However, as Chaston (1972) noted, to date there are no experiments which "completely explain the nature of the linkage between activity and drift". In short, it is still not conclusively known if increased activity causes the animals purposefully to enter the water column, or whether drift is a passive process caused by the accidental dislodgement of the more active specimens. It is possible that the former hypothesis is more acceptable in view of the strong and apparently directed swimming ability of Gammarus. Chaston (1972) noted that "the few benthic invertebrates which are capable of strong swimming, for example gammarids, may intentionally enter drift to move to a new habitat".

In sum, it is clear that light depresses the activity of G. pseudolimnaeus and that there is a marked seasonal and diel pattern of activity. However, factors such as current, light and food may also exert a significant influence on activity. This suggests that a hierarchy of factors may influence activity, or drift, patterns.

6.4.4 The Influence of Substrate on Activity

In our experiments, chi-square analysis indicated activity was significantly higher during daylight with current running ($P \leq 0.1$) and stopped ($P \leq 0.1$) when there was no susbstrate in the tanks (Table I). This may be a result of an unsuccessful search by gammarids for darker areas of stream normally found in the substrate.

There was no significant difference in activity at night ($P \leq 0.75$) in the presence or absence of a substrate when the current was running. However, there was significantly less nocturnal activity ($P \leq 0.025$) when there was no substrate and the current was stopped. This lower level of activity is puzzling, but may have been caused by an easier access to food in the tank when no interference from substrate was encountered.

6.5 SUMMARY

To return to my initial two points about the behaviour of invertebrates as applied to toxicological testing, it is clear that before testing programs can begin, careful attention must be paid to all the possible factors which may influence patterns of behaviour. Only after this "background" or "natural" component has been thoroughly explored, will we be able to begin to make sensible judgements on toxicological evaluations. In so doing, I believe that we shall frequently discover that behaviour among invertebrates is often more complex than was initially thought. This is not to deny the potential usefulness of invertebrates in toxicological tests of "screening programs". However, unless close attention is paid to obtaining a thorough picture of all the factors which may influence behaviour or constitute stress to aquatic invertebrates, our conclusions about toxicological stress may not only be glib but quite misleading - especially if extrapolations from the laboratory to the field are attempted, as in the case of effluent regulations of the Environmental Contaminants Act.

6.6 ACKNOWLEDGMENTS

I would like to acknowledge the close collaboration of Drs. H.B.N. Hynes and N. Kaushik in this work which was funded in part by N.R.C.

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6.8 QUESTION PERIOD

Peter Dill: You claim that the onset of activity was stimulated by darkness. What is your basis for this claim?

Ron Wallace: This was based on repeated experiments which indicated light to be a controlling factor for activity, and previous literature.

Don McLeay: During your seasonal effects studies, were your test and acclimation temperatures constant?

Ron Wallace: Yes, they were identical to that in the field at that time of year.

Wayne Wager: Did you alter your photoperiod to coincide with the natural photoperiod?

Ron Wallace: Yes, all factors were so adjusted.

A FIELD TECHNIQUE FOR STUDYING THE AVOIDANCE OF FISH TO POLLUTANTS

IAN K. BIRTWELL*

Birtwell, I.K. 1977. A FIELD TECHNIQUE FOR STUDYING THE AVOIDANCE OF FISH TO POLLUTANTS. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5-AR-77-1, Halifax, Canada. pp. 69-86.

An experimental field technique is described which may be used to study the behavior of fish in shallow, stratified waters. The technique is particularly suited to determining the avoidance or preference, by fish, of water quality changes in both polluted and unpolluted situations.

The technique was used to determine the effects of pulpmill effluent on the vertical distribution of juvenile salmon *(Oncorhynchus keta* and *Oncorhynchus tshawytscha)* and juvenile herring *(Clupea harengus pallasi)* in a brackish water environment. Although there was a differential response, all species of fish reacted to changes in the receiving water quality which were associated with the discharge of pulpmill effluent. Avoidance effects increased with proximity to the outfalls from the pulpmill.

Birtwell, I.K. 1977. A FIELD TECHNIQUE FOR STUDYING THE AVOIDANCE OF FISH TO POLLUTANTS. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5-AR-77-1, Halifax, Canada. pp. 69-86.

Le document décrit une technique d'étude sur le terrain qui permet d'observer le comportement des poissons dans une nappe d'eau stratifiée peu profonde. La méthode sert particulièrement à déterminer l'attirance ou la répulsion de ces animaux à l'égard de certaines modifications de la qualité de l'eau, tant polluée que saine.

La méthode a servi, en particulier, à contrôler les effets de l'effluent d'une usine de pâte à papier sur la distribution verticale des saumoneaux *(Oncorhynchus keta* et *Oncorhynchus tshawytscha)* ainsi que des jeunes harengs *(Clupea harengus pallasi)* dans un milieu saumatre. On a noté une réaction variable; toutefois, les trois espèces ont modifié leur attitude selon la qualité de l'eau ambiante dans le voisinage du déversement de l'effluent de l'usine. Le comportement de répulsion s'est accentué en fonction de la proximité du lieu de décharge.

7.1 INTRODUCTION

Certain water quality variables have been identified as causal factors in the migration of juvenile salmonids into estuaries and the marine environment (Baggerman, 1960; McInerney, 1964; Mason, 1974; Hoar, 1976). The residence time of juvenile salmonids within estuaries is related to the species and ambient water quality variables. The latter plays

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an important role in the direction of migration, and acclimation prior to the marine phase of their life cycle (Houston, 1957; Baggerman, 1960; McInerney, 1964).

Many estuarine and nearshore coastal areas in British Columbia support industrial operations which discharge their effluent into the aquatic environment. Some of these industrial operations are associated with the forest products industry, effluent from which has been shown to affect juvenile salmonids under laboratory conditions. Lethal and sublethal effects have been documented (Alderdice and Brett, 1957; Sprague and Drury, 1969; Davis, 1973; Webb and Brett, 1972; McLeay, 1973; Howard, 1975) and some of the responsible variables identified (Leach and Thakore, 1973; Rogers *et al*, 1975). A review of relevant information has been made by Walden (1976).

There have been few field studies on the effects of pulp mill effluent on juvenile salmonids. However, the results of these studies have inferred that juvenile salmonids may avoid areas of water quality degradation (Fujiya, 1961; U.S. Dept. Interior, 1967; Livingston, 1975).

Laboratory studies on the avoidance and preference reactions of fish to natural water quality variations and pollutants have been carried out by a number of researchers (e.g. Jones *et al*, 1956; Höglund, 1961). Two basic laboratory experimental techniques have been used to create either a horizontal or vertical gradient in water columns. The effects of pulpmill effluent on salmonids has been studied using the former apparatus (Sprague and Drury, 1969; G. Greer - pers. com.) while the effects of variation in salinity, temperature and light have been studied by Hurley and Woodall (1968), Brett (1952) and Hoar *et al* (1957) respectively using a vertical gradient apparatus.

These studies on behavior have provided valuable information regarding salmonids but of equal importance is the determination of the effects of the same variables under field conditions. The synergistic or antagonistic effects of certain physical, chemical and biological variables within the environment may substantially modify the effects demonstrated under laboratory conditions. Hence, there is a major requirement for field studies in ecological research: a topic which was stressed by Connell (1974).

The requirement of migrating salmonid fry to acclimate to salinity and other variables in the marine environment may initially restrict them to the upper, brackish water layers of vertically stratified estuaries. Most pulpmill effluent discharges are close to, or rise to, the upper layers of the receiving waters depending on many factors such as the nature of the discharge and ambient environmental conditions (Waldichuk, 1974). Thus, in regions of industrialization, the salmonids may encounter lethal and sublethal levels of pollutants. Accordingly, effluent disposal practices may conflict with the needs of juvenile salmonids and change their behavioral patterns.

In view of the paucity of information on the avoidance of juvenile fish to pollutants under field conditions and the need to generate such information from a management viewpoint, an experimental technique was developed to study the effects of natural variables and pollutants on the vertical distribution of juvenile fish under field conditions.

The objective of this study was to determine the applicability of the technique in assessing the effect of Bleached Kraft Mill Effluent (BKME) on the preferred vertical distribution of juvenile chum *(Oncorhynchus keta)* and chinook *(Oncorhynchus tshawytscha)* salmon and juvenile herring *(Clupea harengus pallasii)* in an estuarine environment. The experimental technique is described and a preliminary assessment of data collected between June 28 and July 16, 1976 is presented.

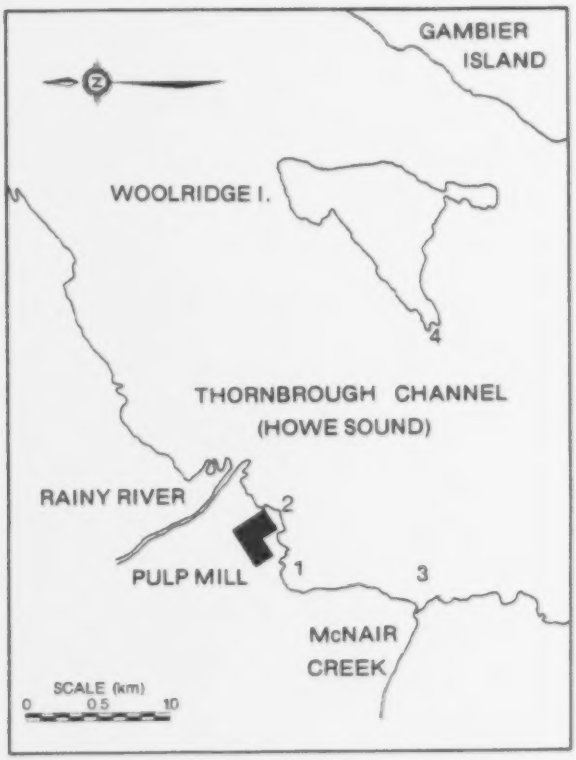

Fig. 1. Map of study area showing experimental sites.

7.2 STUDY LOCATION

The study was carried out in Howe Sound, British Columbia, adjacent to a full-bleach kraft mill which has a daily production capacity of 540 ADT (U.S.) of full bleach pulp. Approximately 27 million gallons of effluent are discharged daily through separate sewers into the surface brackish water layers of Thornborough Channel: 70% of the discharge is from the alkaline sewer, the remaining 30% is from the bleach plant. The highest concentrations of effluent occur in the uppermost water layers. Thereafter, effluent concentrations decrease with depth and distance from the outfalls.

The experimental sites shown in Figure 1 were chosen to encompass, and also to extend, to the southeast, beyond the usual zone of influence of the effluent from the pulp mill thus providing differing water quality conditions. Site 1 was approximately 80 m from both the acid and alkaline sewer discharges while sites 2, 3 and 4 were 350 m, 950 m and 1750 m from site 1 respectively. Site 4, at Woolridge Island, was chosen as the "control site".

The importance of this area to the salmon fishery is emphasized by the average annual spawning salmonid escapement of 390,000 (between 1970 - 1974) into the rivers and creeks which enter Howe Sound.

7.3 MATERIALS AND METHODS

7.3.1 Preference Apparatus

Two basic apparatus were used in the experiments.

7.3.1.1 Apparatus Used in Experiments with Juvenile Salmon

The apparatus was comprised of two components: the preference chambers, and a flotation unit. Pertinent details of the preference chambers are shown in Figure 2 and reference should be made to this figure as required.

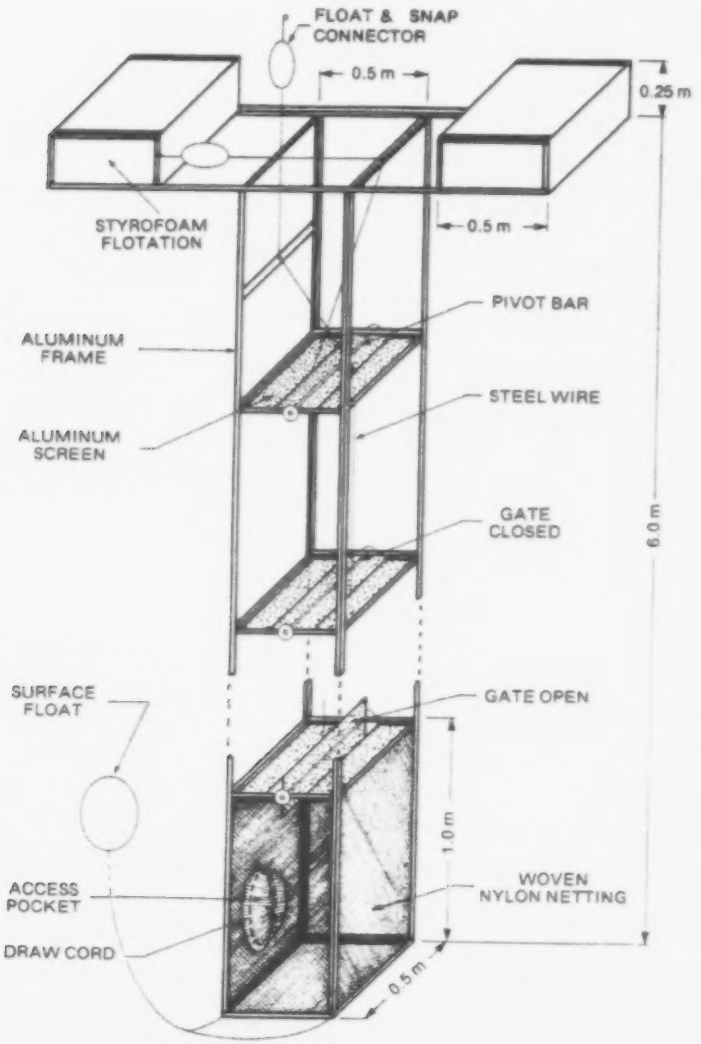

Fig. 2. Diagram of preference apparatus.

The 0.5 X 0.5 X 6.0 m preference chamber framework was constructed with aluminum angle (0.5 X 3.8 X 3.8 cm) which was intersected at 1.0 m intervals with 0.5 X 0.5 m frames of aluminum angle (0.3 X 3.8 X 3.8 cm). Each intersecting frame, located at 1 m, 2 m, 3 m, 4 m, and 5 m subtended a gate mechanism of aluminum screen (0.3 aperture; 42% open area). The gate (0.3 X 18 X 43 cm) was riveted onto a 2 cm diameter aluminum pivot tube which passed through two sides of the intersecting frame, and was retained by washers and a split pin. When closed the gate abutted the adjacent aluminum screen and framework, and upon rotating through 90°, a rectangular opening was created which was approximately 43% of the area of the total partition. The gates were connected to each other using 1.5 mm diameter stainless steel wire which was passed close to the margin and midway along the longer sides of each gate. Brass collars were clamped to the wires below each gate to act as stops, thus limiting the extent of gate movement. In the uppermost chamber the wires were crossed to opposite sides and led through guide holes in the aluminum framework. The wire used to close the gates was led through an aluminum bar 0.5 m above the uppermost gate and then through the main framework. The wire used to open the gates was led directly through the upper framework. Each wire terminated in a float and snap connector which allowed connection to the floatation section and prevented movement of the gates.

The exterior of the preference chambers was covered with woven nylon netting (3.0 mm aperture) which was retained on the framework with an adhesive and nylon twine. A funnel and 25 cm long access pocket of nylon netting were situated in the center of each chamber. A drawcord around the distal portion of the funnel permitted its closure.

A float and a 10 m length of 1.25 cm polypropylene line was attached to the frame at the base of the preference chambers.

The flotation unit was constructed of 0.3 X 3.8 X 3.8 cm aluminum angle which retained two 50 X 50 X 25 cm styrofoam blocks at the extremeties of two 2.1 m sections of aluminum angle. To ensure retention of the blocks, steel strapping was placed around the styrofoam and the framework.

Prior to experimentation the two sections of the preference apparatus were joined with bolts which passed through their respective aluminum frames.

7.3.1.2 Apparatus Used in Experiments with Juvenile Herring

The preference chambers (0.5 X 0.5 X 5.0 m) used in experiments with juvenile herring differed from those used in the experiments with juvenile salmon.

The framework was of steel tubing (2 cm O.D.) with partitions (0.5 X 0.5 m) at 1 m, 2 m, 3 m and 4 m intervals which subtended a gate of nylon netting on a steel tubing frame. The gate was hinged to the metal framework and opened downwards to create an opening which was approximately 10% of the total partition area. Although the size of the openings between chambers in this apparatus was less than that in the other preference apparatus, described above, experiments carried out in 1975 proved that juvenile fish were not impeded from moving between all chambers of the apparatus. Polypropylene rope (1.25 cm diameter) was connected to each gate, and via a pulley located at the base of the chambers was led back to the upper portion. Thus a mechanism for opening and closing the gates utilized one rope.

Flotation of the chambers was provided by two styrofoam blocks retained in a wooden framework bolted to the top of the chamber sections.

7.3.2 Experimental Procedure

Each preference apparatus was moored to a fixed object via the flotation section, and the gates between the partitions were closed.

Ten fish of the same species were removed by net from the holding facilities and placed in each of six labelled buckets of water from the "control" site. (Five buckets of fish were required for the experiments with herring.)

The fish were transported by boat to the experimental site.

At each experimental site the preference apparatus was drawn to the surface using the rope which was attached to its base. Thus the chambers pivoted around the flotation section so that the access pockets were uppermost. Approximately half of the width of the preference chambers was exposed, the rest remained submerged. The access pocket was opened and ten fish were gently but quickly transferred into each chamber of the apparatus. The drawcord around the funnel to the access pocket was used to seal the aperture after entry of the fish. The apparatus was gently lowered into the vertical position in the water column. The snap connector to the wire used to close the gates was removed from its anchor on the flotation unit and the wire used to open the gates was pulled vertically and its snap connector placed on the anchor point on the flotation unit. The time of opening the gates was noted. The fish then had access to the total water column via the open gates.

Immediately after opening the gates water quality determinations were carried out at a depth of 0.5 m and successive 1.0 m intervals to 5.5 m. Water samples were also collected at these depths for subsequent laboratory analysis; the water transparency was recorded.

After a predetermined exposure period (3.0 h.) the gates were closed by operating the appropriate wire in the same manner used to open the gates.

Water quality determinations were carried out employing the same methods (at the same depths) used at the start of the experiment.

The preference apparatus was raised to the water surface and the fish in each chamber were removed by the use of nets, enumerated, and placed in separate labelled buckets. After transport to the laboratory the fish were anaesthetized, re-counted and their length was determined.

To prevent a growth of fouling organisms and the accumulation of detritus on the preference apparatus (which would restrict the flow of water through the netting) it was necessary to brush the exterior and, where convenient, remove the apparatus from the water until the next experiment.

7.3.3 Water Quality

Dissolved oxygen, temperature, conductivity, pH, oxidation-reduction potential (ORP) and depth were determined with an Hydro Products multiprobe sensing unit. This apparatus was calibrated daily using standard solutions for pH, conductivity and oxidation-reduction potential. Dissolved oxygen calibration was carried out by standardization against oxygen determinations using the Winkler technique.

Apparent color was determined using the platinum-cobalt technique (American Public Health Association 1975).

Water transparency was recorded using a Secchi disc.

7.3.4 Fish

The chum salmon used in the experiments were obtained from Inches Creek stock (B.C.). The juvenile fish were retained at the Pacific Environment Institute, West Vancouver, B.C. in well-water at 10-10.2 C between February 3 and April 17, 1976. They were slowly acclimated to salt water by April 28, 1976 at a salinity which varied between 23.0 and 28.3 $^{o}/_{oo}$. Temperature varied between 7.6 and 10.0 C. Between May and June the temperature of the sea water fluctuated between 8.0 and 13.0 C. On June 24, 1976 the fish were transferred to a recirculating sea water system on board the research barge L. Pacifica. The water used in the recirculation system had a salinity of 28.3 $^{o}/_{oo}$, pH of 8.0 and the temperature was maintained between 15 and 16 C. Dissolved oxygen levels were between 90 and 100% air saturation.

The juvenile chinook salmon were obtained from the Big Qualicum Hatchery and Rearing Facility on Vancouver Island, B.C. and transferred as fry to P.E.I. on June 9, 1976, where they were kept in well-water until June 20 after which they were acclimated to salt water (23.0-28.3 °/oo, 8.0-13.0 C). A sample of the saltwater acclimated fish was transferred to the L. Pacifica on July 1, 1976.

The juvenile chum and chinook were fed rations of Oregon Moist Pellets until the day proceeding experimentation.

Juvenile herring were caught in an area of Howe Sound adjacent to the study area. The fish were captured in late evening using a 30 m X 2 m beach seine. The fish were held at the "control" site in aluminum cages (1.5 m deep, 2 m diameter) covered with nylon netting having apertures of 0.3 cm. The cages were moored in the surface waters. These fish were retained at the "control" site for at least two days prior to experimentation.

The mean lengths of the fish used during the experiments were:

Chinook	6.47 ± 0.32 cm
Chum	6.52 ± 0.41 cm
Herring	6.03 ± 0.67 cm

7.3.5 Data Analysis

Only a preliminary analysis of the data was undertaken, using Analysis of Variance, Duncan's Multiple Range Test (Steel and Torrie, 1960) and Correlation Analysis as required. More detailed analysis will be carried out in the future in an attempt to determine the effect of single variables and combinations of variables on the vertical distribution of the experimental fish.

7.4 RESULTS

7.4.1 Water Quality

TABLE I Average water quality data at sites 1 - 4 and depths 0.5 - 5.5 m.

VARIABLES

DEPTH (m)	TEMPERATURE (C) SITE				SALINITY (°/oo) SITE				OXYGEN (mg l^{-1}) SITE			
	1	2	3	4	1	2	3	4	1	2	3	4
0.5	16.8	16.0	13.0	16.1	6.8	6.7	4.5	8.0	9.8	10.2	11.4	11.6
1.5	16.0	15.7	15.2	16.0	8.4	7.9	7.6	9.1	10.4	10.7	11.4	11.5
2.5	15.8	15.7	15.4	15.5	9.9	9.9	9.4	10.4	10.4	10.6	11.4	11.4
3.5	15.3	15.3	15.2	15.0	12.4	11.4	11.2	12.5	9.9	10.2	10.7	10.9
4.5	14.3	14.4	14.4	14.2	14.9	14.4	14.0	15.4	9.3	9.6	10.2	10.1
5.5	13.5	13.6	13.4	13.2	16.9	16.9	16.4	17.8	8.8	9.0	9.6	9.5

VARIABLES (CONTINUED)

DEPTH (m)	pH SITE				COLOR SITE				ORP (mV) SITE				SECCHI (m) SITE			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0.5	7.8	8.5	8.2	8.5	242.5	133.9	40.7	23.9	126	141	212	256	0.5	0.9	2.1	2.1
1.5	7.6	7.9	8.4	8.5	90.5	60.9	31.1	23.1	106	162	211	260				
2.5	7.6	7.6	8.4	8.5	60.1	43.7	26.4	21.2	83	161	214	263				
3.5	7.7	7.8	8.3	8.4	42.8	35.0	21.8	18.8	68	161	217	267				
4.5	7.8	7.8	8.2	8.3	35.0	24.8	18.3	15.2	58	154	224	266				
5.5	7.7	8.0	8.0	8.1	25.6	19.9	15.7	13.8	52	145	226	282				

The mean results of 744 water quality determinations within the study area are shown in Table 1. This table shows how some of the water quality variables gradually change with distance and depth from the pulp mill outfalls.

7.4.2 Fish Distribution

To facilitate comparison between fish distributions in the preference chambers and water quality data, the number of fish per depth interval will be related to the mid-depth of each chamber of the preference apparatus. For example, the fish distribution within the 0.0 to 1.0 m depth interval will be considered to be at 0.5 m.

7.4.2.1 Grouped Data

The raw data on fish distribution from individual experiments were subjected to Analysis of Variance followed by Duncan's Multiple Range Test to determine if significant differences were evident in the distribution of the fish between sites and depths. A comparative assessment of the grouped data from 40 experiments with chinook, 22 experiments with chum and 18 experiments with herring is shown in Table 2. Histograms representing the mean percentage of fish in the chambers of the preference apparatus at each experimental site are shown in Figures 3, 4 and 5 for chinook, chum and herring respectively.

TABLE 2 Comparative analysis of fish distribution data between sites for each depth using Duncan's Multiple Range Test ($P < 0.05$). Only significant results are shown.

DEPTH (m)	CHINOOK	CHUM	HERRING
0.5	(1,2,4)-(3)	(1)-(2,3,4)	
1.5	(1,2)-(3,4)	(1)-(2,3,4)	
2.5	(1,2,4)-(1,3,4)		
3.5		(1,2,3)-(3,4)	(1) - (4)
4.5			
5.5			NOT APPLICABLE

7.4.2.1.1 Chinook

Juvenile chinook exhibited a significant preference for the 0.5 m water layer at site 3. There was an insignificant difference between the number of fish at this depth at sites 1, 2 and 4. At 1.5 m significantly higher numbers of fish were present at sites 1 and 2 than at sites 2 and 4. With increasing depth the difference in numbers of chinook, between sites, was less pronounced and between 3.5 and 5.5 m significant differences were not apparent.

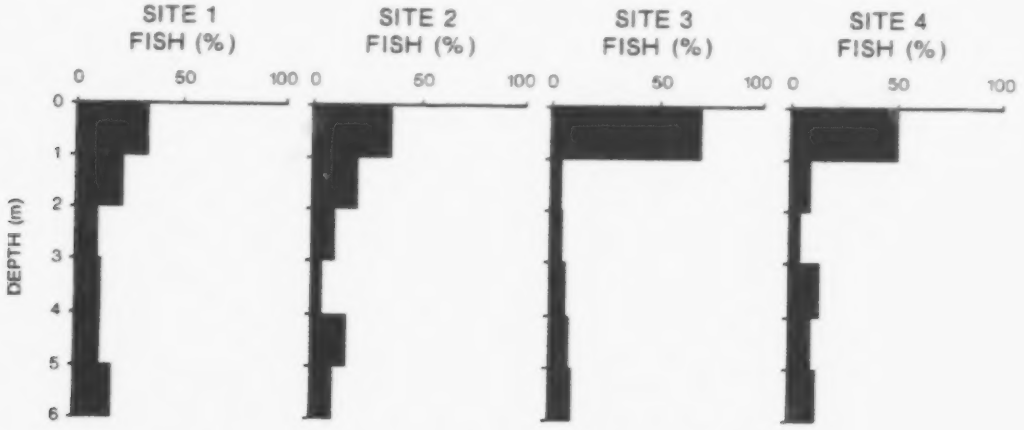

Fig. 3. Vertical distribution of juvenile chinook salmon.

7.4.2.1.2 Chum

Juvenile chum salmon exhibited a marked change in their vertical distribution between sites 1 and 4. At site 1, there were significantly fewer fish at 0.5 m than at sites 2, 3 and 4. The same significant relationship between sites was apparent at 1.5 m but in contrast to the data for 0.5 m, the highest numbers of fish were recorded at site 1. At 3.5 m the number of fish at site 1>2>3>4 but sufficient variability existed to group sites 1, 2 and 3; 3 and 4 as homogenous sub-sets. Insignificant differences between sites were found at other depths.

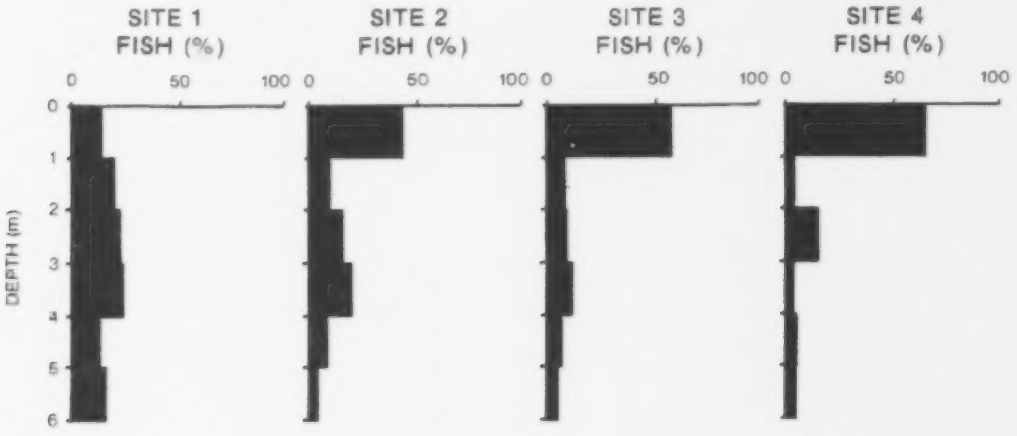

Fig. 4. Vertical distribution of juvenile chum salmon.

7.4.2.1.3 Herring

Studies on the vertical distribution of juvenile herring revealed a significant difference between the experimental sites 1 and 4 only at 3.5 m, where the number of fish at site 1 was significantly less than that at site 4.

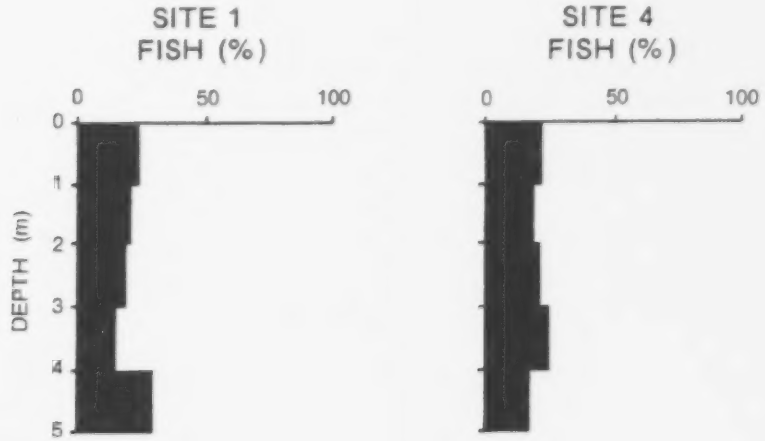

Fig. 5. Vertical distribution of juvenile herring.

7.4.3 Relationships Between Fish Distributions and Environmental Variables

As a preliminary step towards identifying the possibility of relationships between variables and fish distributions, the raw data were subjected to correlation analysis. Temperature, salinity, oxygen, pH, color, ORP, "secchi compensation depth", distance (site), depth, and the mean length of the fish were the variables used. The mean length of the fish was used to determine if size influenced the grouping of the fish. The water quality data were averaged over the 3.0 h experimental period.

TABLE 3 Significant correlation coefficients ($P \leq 0.05$) between species of fish and variables at sites 1 - 4

SPECIES	SITE	n	VARIABLE					
			TEMPERATURE (C)	SALINITY ($^{o}/oo$)	OXYGEN ($mg\ l^{-1}$)	pH	COLOR	DEPTH (m)
CHINOOK	1	60						-0.3329
	2	60		-0.2723		0.4835	0.3188	-0.5197
	3	60	-0.4098	-0.4179			0.6157	-0.5928
	4	60					0.4854	-0.4974
CHUM	1	36						-0.5171
	2	30						
	3	30		-0.6228	0.4836		0.7132	-0.6603
	4	36	-0.5091	-0.5041	0.3883		0.5821	-0.6439

7.4.3.1 Between Sites

Table 3 shows the significant correlation coefficients ($P \leq 0.05$) between species of fish and the above mentioned variables within the water column. A strong depth avoidance by chum and chinook is identified, except at site 1 for chum; thus reinforcing the data presented in Figures 3 and 4.

7.4.3.1.1 Chinook

Numbers of chinook were only correlated with depth (negative correlation coefficient (-)) at site 1, but at site 2, correlation analysis revealed that salinity (-), pH (positive correlation coefficient (+)) and color (+) were significantly related to the fish distributions. At site 3, both temperature and salinity were negatively correlated and color positively correlated with vertical distribution of the fish. At site 4, only color (+) was significantly correlated to the fish distribution.

7.4.3.1.2 Chum

Chum distributions did not show any significant correlation with the variables at site 1, and at sites 2, 3 and 4 depth was negatively correlated with the number of fish. With increasing distance from the outfalls, more variables were identified as influencing the vertical distribution: salinity (-), oxygen (+) and color (+) at site 3. At site 4, temperature (-) was an additional variable which was related to the vertical distribution.

7.4.3.1.3 Herring

Correlation analysis between numbers of herring and the variables at each site did not reveal any significant correlations.

7.4.3.2 Between Depths

In view of the correlation between numbers of chinook and chum with depth and the variation in water quality with depth (Table 1) further correlation analysis was carried out

to determine if relationships existed between the selected variables and the number of fish at each depth in the water column. The results are presented in Table 4. To aid data interpretation, reference should be made to Tables 1 and 2 as appropriate.

TABLE 4 Significant correlation coefficients ($P \leq 0.05$) between species of fish and variables, at depths 0.5 - 5.5 m.

SPECIES	DEPTH (m)	n	VARIABLE							
			TEMPERATURE (C)	SALINITY ($^{o}/oo$)	OXYGEN (mg l^{-1})	pH	COLOR	OXIDATION REDUCTION POTENTIAL (mV)	"SECCHI" (m)	SITE (#)
CHINOOK	0.5	40	-0.5254	-0.3596	0.4840		-0.6366		0.3621	0.4045
	1.5	40			-0.3043	-0.3312	0.6153	-0.3663	-0.4252	-0.4725
	2.5	40				-0.3203				-0.3411
	3.5	40		0.4221						
	4.5	40		0.3460						
	5.5	40								
CHUM	0.5	22	-0.4448		0.5049	0.4272	-0.7979	0.5330	0.7213	0.7408
	1.5	22				-0.5472			-0.4498	-0.6280
	2.5	22		0.4593						
	3.5	22					0.5570	-0.5793	-0.6419	-0.5900
	4.5	22					0.4304			
	5.5	22					0.4668			
HERRING	0.5	18		0.5141						
	1.5	18								
	2.5	18				0.5326	-0.6136			
	3.5	18				0.6113		0.4856	0.4832	0.5675
	4.5	18				-0.7430	0.6082	-0.6210		

Significant differences between sites at each depth for distributions of chinook, chum and herring were presented in Table 2. The significant correlation coefficients between number of fish and the variable "site" (equivalent to distance) in Table 4, relate to those inferences which were identified in Table 2. At 0.5 m, chum and chinook were found in higher numbers with increasing distance from the outfalls whereas at 1.5 m and 2.5 m, a decreasing trend was identified for numbers of chinook with distance. A similar decreasing trend was identified for chum at 1.5 m and 3.5 m. The numbers of herring showed a significant increase with progressive distance from the outfalls at 3.5 m only.

7.4.3.2.1 Chinook

There was a negative correlation between numbers of chinook and temperature, salinity and color, but a positive correlation with oxygen and "secchi" at 0.5 m. These correlations infer the significant preference of chinook for lower temperatures and salinities at site 3, and water of lower color, higher oxygen and increased water transparency which occurred with progressive distance from the outfalls. At 1.5 m there was a negative correlation between oxygen, pH, ORP and "secchi" with fish number, indicating the higher numbers of fish which were found in this water layer with proximity to the outfalls and also the increase in values of these variables with distance from the outfalls. The positive correlation with color similarly indicates the presence of higher numbers of fish with approach to the outfalls, reflecting the downward shift of the fish from surface waters, rather than a direct preference of highly colored water (effluent).

At 2.5 m higher numbers of chinook were found in the waters of lower pH close to the outfalls. Again, this possibly reflected the downward shift in the fish from the surface waters.

Higher numbers of chinook were associated with higher salinity water at 3.5 m and 4.5 m, however there was not a significant difference in this variable between sites, nor in the distribution of chinook between sites at these depths.

7.4.3.2.2 Chum

The number of chum salmon recorded at 0.5 m were positively correlated with oxygen, pH, ORP and water transparency, values of which increased with distance from the outfalls. In contrast, there was a negative correlation between number of chum and temperature and color.

At 1.5 m values of pH and "secchi" were negatively correlated with the number of chum. Values of both variables increased with distance from the outfalls and there was a concomitant decrease in the number of fish with distance indicating the selection of particular depths by fish, between sites. At 2.5 m only salinity was positively correlated with the number of chum, whereas at 3.5 m there was a positive correlation between the number of chum and color reflecting the increase in fish numbers at depth closer to the outfalls. In contrast ORP and "secchi" were negatively correlated with fish numbers. Significant positive correlations existed between numbers of chum and color at 4.5 m and 5.5 m.

7.4.3.2.3 Herring

Significantly different distributions were not obtained between the number of herring at sites 1 and 4, depth 0.5, but there was a positive correlation between fish number and salinity values.

At 2.5 m a positive correlation with pH and a negative correlation with color existed, similar to that shown for chum at 0.5 m.

At 3.5 m higher numbers of herring occurred at site 4 than at site 1 and positive correlations were identified with pH, ORP and "secchi", values of which also increased with distance from the outfalls.

An insignificant difference occurred between the mean numbers of herring at sites 1 and 4 at 4.5 m (Table 2) but the individual recordings of fish at this depth were found to be negatively correlated with pH and ORP, and positively correlated with color.

7.5 DISCUSSION

7.5.1 Water Quality

Changes in water quality variables with distance from the pulp mill outfalls were manifest in a reduction in water transparency, dissolved oxygen and ORP values, and an increase in color with proximity to the outfalls. Although pH variations were noted to be erratic between specific experiments, mean values showed a relative depression close to the outfalls even in the more saline waters.

The natural vertical stratification of the receiving waters resulted in the effluent remaining in the upper water layers, a situation which was most apparent within about 350 m of the outfalls.

Some of the recorded variations in water quality were not a direct result of effluent discharges. For example, the low values recorded for ORP (variation in which is an indication of a reducing or oxidizing environment) may have been associated with the degradation of fibre beds close to the outfalls which release "reduced substances" into the water column.

7.5.2 Vertical Distribution of Fish

In view of the variation in water quality over the study area, it may be expected that the experimental fish would show some preference for water of a particular quality.

Variables such as salinity, that have been identified as factors in the seaward migration of juvenile salmon (McInerney, 1964) may exert an effect on this preference.

McInerney (1963) indicated that the salinity preference or tolerance of chum and chinook salmon increased during the spring months, and that maximum schooling activity occurs at a time when the fish would be moving through an estuary, a statement which was reinforced by Hoar (1976). It is obvious from the data we obtained, especially at sites 3 and 4 that both chum and chinook preferred the upper 0.5 m waters of the estuarine water column. It is probable that this behavior is accompanied by schooling. However, such results would appear to be erroneous on the basis of known associations of the fish with water quality variables and some of the published information. The salmonids were acclimated to lower temperatures and higher salinities than those encounted in the uppermost surface waters of the study area, with the exception of the lower temperatures at 0.5 m by McNair Creek (site 3). Therefore, it was expected that the fish would select the lower water layers where water quality variables approached those at which the fish were acclimated. Mason (1974) also noted a similar discrepancy in the recorded behavior of chum fry between field and laboratory studies. Mason found that juvenile chum are highly adaptable and will seek environmental conditions best suited to their needs. This deduction was demonstrated by their ability to move into fresh or marine waters on a regular basis suggesting, as Weisbart (1968) found, that chum fry are euryhaline. Thus Houston's (1957) comment that it is improbable that chum fry would return to "freshwater" following contact with sea water is disproven by field observations.

Hoar *et al* (1957) showed that schools of chum fry had a marked preference for light. During our field studies water transparency, as determined from "Secchi disc" recordings was greatest at those sites where lower color values were recorded. Higher numbers of chum and chinook were recorded in the surface (0.5 m) layer at these sites, possibly inferring a relationship with light.

Chinook salmon fry are known to be stenohaline but possess a strong tolerance of high salinities (Weisbart 1968). In addition, their capacity for rapid acclimation to salinity distinguishes the species from other salmonids (Hoar 1976). In view of their adaptability, it is not suprising that chinook were found in the upper water layers. Nevertheless, it was expected that the chinook would prefer water quality conditions approximating to those which they encountered during the laboratory acclimation period.

The lethal temperature levels for juvenile salmonids which were identified by Brett (1952) never prevailed within the study area, and dissolved oxygen values, although depressed close to the outfalls, were never depressed to levels which would be considered detrimental to the fish (using the criteria supplied by Davis 1975).

Although these basic water quality variables (temperature, salinity and dissolved oxygen) never attained lethal levels, synergistic or antagonistic effects may have influenced the distributions of the test fish.

Herring did not show a marked vertical distribution pattern and at the "control" site tended to remain evenly dispersed over the 5 m experimental column. It is unlikely that temperature, salinity or dissolved oxygen levels were limiting factors in the distribution of these fish, which were collected from surface waters of Howe Sound. Furthermore, McMynn and Hoar (1953) found that the larvae of the herring could tolerate a wide range of salinities (6.06 $^{o}/_{oo}$ to 34.28 $^{o}/_{oo}$).

Thus it was demonstrated that juvenile chum and chinook salmon displayed a distinct, significant preference for the uppermost 0.5 m water layers in the less contaminated regions of the study area. Between 50 and 70% of the experimental fish were found in the

0.5 m water layers at sites 3 and 4. Herring did not exhibit the same response, and were seemingly indifferent to the variations in water quality at the "control" site.

7.5.3 Changes in the Vertical Distribution of Fish

The vertical distribution of chinook and chum salmon changed with proximity to the outfalls where more fish were found at greater depths. At site 3, in addition to the effects of other variables, the colder, less saline surface water adjacent to McNair Creek was preferred by the salmon to the warmer, more saline water typical of their acclimation conditions. However, the vertical distribution pattern of the salmon changed with proximity to the outfalls, and suggested that the fish were avoiding the upper 0.5 m water layers.

The vertical distribution of chinook showed that significant changes occurred at distances up to 350 to 950 m from the outfalls. This deduction is based upon the presence of numbers of fish at 1.5 m depth at sites 1, 2 in contrast to the lower numbers of fish recorded at this depth at sites 3 and 4.

The vertical distribution of chum salmon was most significantly changed in the surface water layers to a depth of 1.5 m, at the site closest to the outfalls. The inference was that the distribution of chum salmon was significantly affected up to a distance between 80 and 350 m from the outfalls.

The numbers of herring were significantly different at 3.5 m depth between sites 1 and 4. Higher numbers of herring were found at the "control" site perhaps indicating a shift in the preferred vertical distribution pattern at the site closest to the outfalls. The data from specific experiments showed that the vertical distribution of herring could be markedly changed close to the outfalls from the pulp mill; the herring avoiding the uppermost water layers.

The mean vertical distribution patterns of the experimental fish over the study area, are useful in defining the approximate extent of the effects due to environmental changes, and accordingly should be assessed with caution. During the study period, the pulp mill did not operate continuously for two days and as a result only a small volume of effluent was discharged. This discontinuity of operation occurred during experiments with chinook and the mean vertical distribution patterns may have been slightly different to those which would have been recorded under maximum operating conditions.

Although the mean data revealed the existence of significant changes in the vertical distribution of the salmon with proximity to the outfalls, individual experiments showed that deviations from the preferred depth ranges of all the species tested can be extremely marked.

7.5.4 Relationship Between Fish Distribution and Water Quality

In view of the identified significant preference of the salmonids for the uppermost water layers, and the inference from the water quality data that components of pulp mill effluent were in the same layers, it is not surprising that significant positive correlations were found between the numbers of salmonids in the 0.5 m water layer with progressive distance from the outfalls. The increase in the number of fish at 1.5 m and below reinforces the implication of an avoidance reation to the surface waters containing pulp mill effluent. Jones *et al* (1956) and Dimick *et al* (1957) found that juvenile chinook elicited avoidance reactions to kraft waste at about 2.5 to 5% dilution, and also the inference of a preference reaction at more dilute concentrations. Such a response variation in dilute concentrations would tend to detract from any determination of a response threshold, and may help to account for the very broad threshold effect (0.001 - 10%) which Sprague and Drury (1969) recorded for the avoidance response of juvenile Atlantic salmon to BKME.

The dilutions of pulp mill effluent in the receiving waters were not determined in this preliminary study, but instead reliance was placed upon very approximate estimations of effluent and the secondary effects of the effluent on basic water quality variables. To this extent, direct comparisons between reported laboratory results and the field observations would not be practical. However, the results of correlation analysis inferred a relationship between certain water quality variables, attributable to the discharge of pulp mill effluent, and the salmonids. The negative correlation between color and the abundance of salmon in the 0.5 m water layer illustrates this point. Both chinook and chum were found in lower numbers in waters with high color values.

Herring elicited a negative response to color at 2.5 m depth which was similar to that shown by salmon in the surface waters. The presence of color at this depth was far less intense than in the surface waters. This correlation at 2.5 m depth may have been fortuitous, or alternatively the herring were extremely sensitive to low concentrations of effluent and greater concentrations may have resulted in chemosensory blockage.

During in-situ bioassay experiments (unpublished data) juvenile herring were more sensitive than either chum or chinook salmon to the adverse water quality variations found within the study area. It was expected that marked avoidance reactions to the pulp mill effluent would be exhibited by the herring due to this sensitivity. The results did not confirm this hypothesis but suggested that some chemosensory effect may be influencing the herring and to a lesser extent, the salmonids in high concentrations of effluent. Höglund (1961) concluded that avoidance reactions of fish to sulphite waste liquor (SWL) diminished in steep concentration gradients. Fish with a well developed olfactory sense avoided low non-toxic concentrations of SWL but in high toxic concentrations of SWL the avoidance reactions diminished. If such a reaction occurred during the reported studies in Howe Sound correlations between fish abundance and water quality variables would be difficult to ascertain. In this context, the absence of a general vertical distribution change by herring to "adverse water quality" close to the pulp mill outfalls may have been related to blockage of the sensory powers of these fish.

The ecological significance of avoidance reactions has been discussed by a number of authors (e.g. Höglund 1961) and there seems to be a concensus of opinion that such reactions favour the survival of the species. This of course, presumes that the avoidance reactions result in a movement into regions of more acceptable water quality. Juvenile salmon school upon entering estuaries, a behavioral pattern which presumably enhances their survival. In the laboratory BKME interrupted this behavior (Walden and Howard 1968) and the results of this field study tend to support those findings. Surface aggregations of the salmonids being vertically dispersed close to the pulp mill outfalls. Thus a conflict was apparent as the necessary schooling behavior of the fish was interrupted by the avoidance of adverse water quality.

Field studies on the distribution of fish in the vicinity of pulp mill discharges have reached different conclusions. A report by Van Horn (1961) mentions studies which did not find avoidance reactions by fish whereas conclusions to the contrary are to be found in the publications by the U.S. Department of the Interior (1967) and Livingston (1975). Quite obviously variations in the environmental conditions of the study areas and the nature of the pollutants could account for such conflicting viewpoints, but nevertheless, the need for further field studies is emphasized.

In summary, the technique described above, has shown that juvenile salmon exhibited varying degrees of avoidance of surface waters with increased proximity to the pulp mill outfalls. Concommitant water quality determinations enabled a preliminary assessment of the causal factors related to the change in the vertical distribution of the experimental

fish. The technique would seem particularly suited to those field situations where effluent is discharged into surface waters frequented by fish and where there is a need to document the zone of influence. However, the determination of those factors responsible for avoidance reactions exhibited by fish in the field will remain difficult because of the interactions between environmental variables.

7.6 ACKNOWLEDGEMENTS

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7.8 QUESTION PERIOD

John Davis: Your observation on the herring is facinating. Most of the kills we have had around B.C. pulp mills seems to be herring rather than salmonids. I wonder if in some cases there is an inability to detect the effluent in surface waters resulting in mortality.

Ian Birtwell: It is very probable that herring respond to the decreased light intensities beneath a dark colored pulp mill effluent plume, by dispersing within the water column, as

they would at dusk in response to the decreasing light intensity and diurnal movements of their food. Accordingly, some herring may enter high concentrations of the effluent. If the effluent is acutely toxic and the fish effect an orthokinetic response rather than a chemotaxis, it may be expected that some fish would die. Alternatively, if the fish had their sensory powers blocked by the effluent, the same final result may be expected. Certainly, other workers have concluded that juvenile herring may have their sensory powers blocked by high concentrations of a pollutant, a feature which does not confer a high survival potential on the species in regions of high concentrations of toxic effluent. Conversely, if the herring were able to detect extremely low concentrations of effluent, avoidance reactions could promote survival. However, this presumes that other environmental factors would not override the avoidance reactions.

In contrast to salmon, herring appear to be more sensitive to pulp mill effluent. In-situ bioassays which were carried out at distances from the outfalls of the Port Mellon pulp mill resulted in a more rapid mortality of herring in contrast to chinook and chum salmon of the same size.

Hence it is probable that a number of factors are responsible for the death of herring around B.C. pulp mills, but their high sensitivity to pulp mill effluent and their behavior may be major contributing factors.

Gary Vigers: We have also observed greater sensitivity of herring over salmonids with certain toxicants. I wonder if the vertical migration of herring rising to feed at dawn and dusk might overpower their response to the effluent.

Ian Birtwell: It is my understanding that adult herring rise to the surface at dusk in response to a number of factors, light intensity and food being two of these. Young larval herring have been shown to be positively phototactic towards high light intensities, and aggregate at the surface during daylight. However, at night these larval herring disperse in the water column.

It is likely that the stimuli responsible for the diurnal movements of the fish may override the stimuli from pollutants. Such an effect was hypothesized to explain the reactions of larval herring subjected to dilutions of oil dispersants in surface waters. The fish did not avoid the dispersants hence it was presumed that the fish were lacking a chemotactic response or that this response was readily sublimated to the drive of positive phototaxis.

Don McLeay: The apparent increase of sensitivity of herring to toxicants may be attributable to their greater sensitivity to handling stress.

Ian Birtwell: There is no doubt that the herring were stressed during capture with a beach seine. After capture, the fish swam frantically around the periphery of the holding buckets. However, these fish were quickly transferred to large holding cages for at least 2 days prior to any experimentation. During this time their behavior did not indicate that they were highly stressed. Subsequent handling prior to experimentation did not give rise to the same level of response as noted upon capture. This may have been due to the lower densities of fish being transferred in holding buckets, but it is obvious that the herring and salmonids would be stressed by our experimental procedures, and hence their sensitivity to toxicants may have been increased. Unfortunately, this is a concern inherrent in many experiments and should receive greater attention.

ASSESSMENT OF THE TOXICITY OF LANDFILL LEACHATES BY THE RESIDUAL OXYGEN BIOASSAY

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Vigers, G. A. and B. M. Ellis 1977. ASSESSMENT OF THE TOXICITY OF LANDFILL LEACHATES BY THE RESIDUAL OXYGEN BIOASSAY. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5-AR-77-1, Halifax, Canada. pp. 87-106.

The Residual Oxygen Bioassay is a rapid bioassay procedure which for rainbow trout *(Salmo gairdneri)* is sensitive to toxic materials at concentrations in the 96-h LC50 range. The procedure requires less than eight hours to complete and is based on the consumption of available oxygen by fish in sealed containers. A simple method of graphing Residual Oxygen Bioassay results is described to determine threshold values. The procedure was evaluated using sodium pentachlorophenate and landfill leachates. The threshold values obtained from the rapid procedure were directly comparable to static 96-h LC50 bioassays obtained by standard methods.

Regression analysis of Residual Oxygen Bioassay data obtained in the field demonstrated a correlation with duplicate samples subjected to static 96-h LC50 bioassays in the laboratory. Further, there was no significant difference in the response of residual oxygen bioassays controls conducted in the lab and in the field. Application of the procedures to field situations, and the effects of procedural variables such as temperature, fish loading density and pH are discussed. The mobile support facility used for the field studies is also described.

Vigers, G. A. et B. M. Ellis 1977. ASSESSMENT OF THE TOXICITY OF LANDFILL LEACHATES BY THE RESIDUAL OXYGEN BIOASSAY. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5-AR-77-1, Halifax, Canada, pp. 87-106.

L'application de la methode de l'oxygene residuel aux essais biologiques est rapide. La truite arc-en-ciel *(Salmo gairdneri)*, soumise a cette epreuve, se montre sensible a l'echelle des concentrations toxiques LC50* en 96 h. Le processus se deroule en moins de huit heures et se fonde sur la quantite d'oxygene disponible que le poisson peut consommer dans un reservoir hermetiquement ferme. L'etude expose une methode simple de representer graphiquement les resultats de l'essai biologique dans le but de determiner les valeurs limites du seuil de toxicite. On a evalue la methode a l'aide de pentachlorophenate de sodium et d'eaux de lixiviation d'un depotoir. Les valeurs limites du seuil de toxicite obtenues par ce procede rapide se comparaient directement a celles resultant de tests biologiques statiques du LC50 en 96 h realises par des procedes standards.

L'analyse de regression des donnees obtenues sur le terrain par la methode de l'oxygene residuel a revele une correlation avec les essais biologiques du LC50 en 96 h realises en laboratoire a partir d'echantillons analogues. En outre,

*EVS Consultants Limited, New Westminster, B.C. V3M 5R1.

on n'a note aucune difference importante dans le deroulement des experiences temoins d'oxygene residuel effectuees tant en laboratoire que sur le terrain. Le document commente l'application des methodes aux situations rencontrees sur place et les effets de certaines variables liees au procede, telles que la temperature, la densite des populations de poissons et le pH. L'facilite mobile utilize pour les etudes dans le champ est aussi decris.

*Lethal Concentration 50 percent (niveau de concentration toxique provoquant 50 p. 100 de deces).

8.1 INTRODUCTION

The use of toxicity assessment procedures to determine the acceptability of industrial effluents for discharge is now recognized by Federal and industrial bodies alike (Pessah, 1975; Stephan, 1975). There is a real need to conduct in situ or field assessments of the existence, extent and nature of toxic aspects of effluents and leachates, and how these aspects might vary with time and dilution. Because of the potentially large number of samples required in initial surveys, laboratory programs to assess toxicity can be excessively time consuming and costly in terms of manpower, even with simplifications of the 96-h LC50. Carrying regulatory procedures to the field with a mobile laboratory is possible but again expensive in terms of time and equipment. Further, the rate of data generation by these methods may satisfy regulatory requirements but they cannot provide the day to day toxicity information necessary to form a basis for regulations development, nor are they adequate to provide information on patterns and attenuation of toxicity in the receiving environment.

A rapid toxicity assessment procedure, that would correlate with standard bioassay procedures and could be used in the field as a screening test to select those effluents or leachates of interest for stringent testing, would greatly facilitate the definition of environmental problems associated with contaminant discharge.

Previous studies (Carter, 1962; Ballard and Oliff, 1969; McLeay, 1976; Vigers and Maynard, 1977) have demonstrated that the residual oxygen bioassay merits high priority as a rapid test for monitoring purposes. The test is based on the amount of oxygen utilized by rainbow trout in the presence of a toxic material. When oxygen is limited, as in a sealed BOD bottle, the dissolved oxygen remaining at death is directly proportional to toxicant concentration. The consensus of previous studies was that this bioassay can be conducted in less than eight hours with less than 4 liters of effluent, and with a sensitivity comparable to that obtained in static 96-h LC50 tests.

Accordingly, the major purpose of the present work was to compare the results of lab and field work to determine whether the residual oxygen bioassay could be used to assess the existence and extent of toxicity in leachates at the Richmond Landfill site, Richmond, B.C.

8.2 MATERIALS AND METHODS

8.2.1 Site Location and Characteristics

The Richmond Landfill occupies 365 acres of which 100 acres has been utilized adjacent a drainage ditch and the north bank of the Fraser River in the Municipality of Richmond, B.C. Demolition material, wood waste and office waste comprise 95% of the landfill. Putrescibles comprise the remaining 5%. Wastes are segregated with wood wastes being a primary mat. Construction materials are compacted onto the wood wastes and a sand

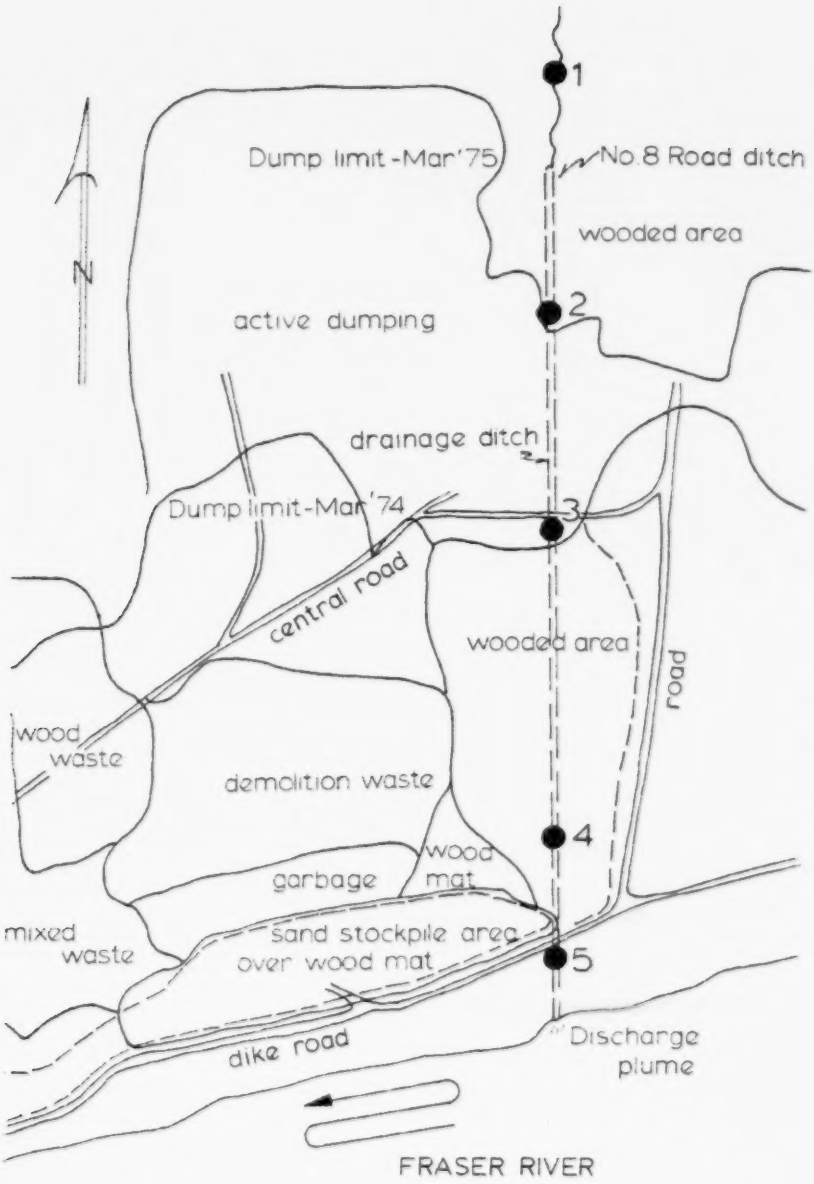


Figure 1. Location of Richmond landfill site.
Scale: 1 inch = 300 feet

layer is added to seal the material. Putrescibles are divided into cells to avoid fire hazards and covered with sand.

The landfill is situated on a peat bog ranging in thickness from 5 feet to 25 feet underlain by sand to silty soil. The site receives approximately 45 inches of precipitation per annum. Runoff from the area is picked up in a shallow ditch, approximately two feet deep, referred to as the #8 Road drainage ditch, which bisects the existing landfill and drains to the Fraser River. While tides may temporarily reverse the direction of flow in the ditch, the leachate inevitably runs into the Fraser River.

8.2.2 Sampling Procedures

For the present study, five sample stations were located along this ditch. Ten gallon grab samples were collected in two 5 gallon plastic containers at each selected sample station. In addition, for the first two series of samples collected, 2 - 1 liter samples for chemical analysis were obtained in plastic bottles at each site. Samples were cooled to 15 ± 1°C if they were to be used immediately in field bioassay, otherwise samples were stored at 4°C until use. Samples for chemical analysis were transported immediately to the Water Quality Laboratory of the Environmental Protection Service, Pacific Region. Dissolved oxygen (DO), water temperature and pH were recorded at the time of sampling.

8.2.3 Bioassay Procedures

Bioassays were conducted with 0.6 - 2.0 g rainbow trout. The fish stocks used in this study were a domestic hybrid strain of rainbow trout, obtained from a commercial source, Sun Valley Trout Farm of Mission, B.C. Mortalities in the holding tanks were less than 0.1% per week throughout the study.

On the day of field testing, the required number of animals (usually 100) were transferred in their own holding tank water (pH 6.2 - 6.8, 4-10 ppm hardness) to an insulated constant temperature chamber in the mobile bioassay lab. The fish were then transported under oxygen to the field for on-site bioassays. Leachates did not require pH adjustment with HCl or NaOH prior to introducing test fish. The temperature maintained during bioassay was that of the holding tanks (15 ± 1°C). Samples were aerated prior to bioassay to ensure that initial dissolved oxygen levels were greater than 90% saturation. Fish were not fed for 48 hours prior to starting the bioassays nor for the duration of the test. One or more control vessels of dechlorinated tap water was run for each bioassay.

Residual oxygen bioassays were carried out using serial dilutions of effluent in BOD bottles (300 ml), as described by Vigers and Maynard (1977). A selected number of test fish, as determined by their size, were placed in each bottle. The bottles were stoppered with a water seal and incubated until all the fish in a test bottle had expired. Each bottle was removed from the bath after 100% mortality and the dissolved oxygen was measured using a YSI model 54 oxygen probe or Delta Oxygen probe with stirrer, calibrated each morning.

To provide an initial screening of leachates for toxicity, two controls and three replicates were conducted at each site on 500 ml aliquots of leachate sample as follows:

- 1 control for each site consisting of air-saturated laboratory dilution water (transported to the field)
- 1 control for each site consisting of 100 percent leachate and no fish (to test oxygen demand of the leachate)
- 3 replicates for each site consisting of 100 percent effluent with 2 fish per bottle

Graphed estimates of residual oxygen threshold values were obtained by plotting residual

dissolved oxygen levels against leachate concentration on double logarithmic paper, resolving the data into two groupings, and selecting best fit straight lines by eye (Vigers and Maynard, 1977). Alternatively, where calculated values were desired, the method of least squares (Miller and Freund, 1965) was used. Raw data were transformed into logarithmic forms and the values solved for curves have the form of a power curve as expressed by equation (1).

$$Y = a\,X^{b} \text{----------} (1)$$

where X is the toxicant concentration in percent or ppm

Y is the residual oxygen concentration in ppm

a is the Y intercept

b is the slope function of the curve. This indicates how Y changes with changes in X.

The threshold value, expressed as percent effluent concentration can be obtained by substituting control values of X into the regression equation.

Static 96-h LC50 bioassays were conducted in 20 l of test solution in all glass containers. Dissolved oxygen and pH levels were measured every 24 hours. To initiate the bioassay 10 fish were added to each tank resulting in a fish loading density of 0.5 - 1.0 gl^{-1}. Percent survival was observed at 30 minutes and 1, 2, 4, 8, 24, 72 and 96 hours. Incidental observations of mortalities at other times were also recorded. 96-h LC50's were estimated by graphical interpolation according to standard methods (A.P.H.A., 1976). When data permitted, 96-h LC50's were calculated by fitting a line to log-probit data, expressed as concentration vs percent mortality at 96 h, according to the nomographic procedures of Litchfield and Wilcoxon (1949).

The reference toxicant for bioassays was sodium pentachlorophenate, prepared from Reagent Grade 2, 3, 4, 5, 6 - pentachlorophenol (M.W. 266.36) by addition of NaOH according to the method of Alderdice (1963).

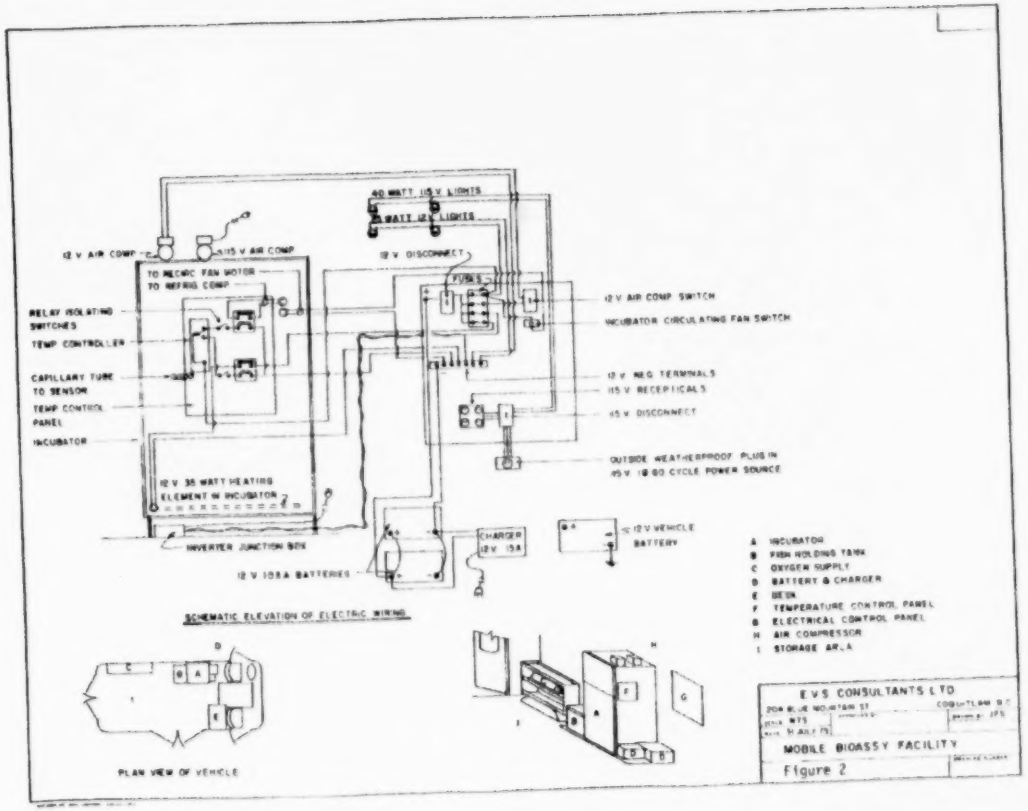
8.2.4 Mobile Bioassay Laboratory

The basic functions of the mobile bioassay laboratory were essentially those required of a fully equipped wet laboratory, with the emphasis being on a pragmatic approach to obtaining toxicity data in the field. The major criteria were constant temperature control for fish holding and bioassay, power supply, mobility and degree of independence of the home lab. Based on these criteria, a mobile lab for residual oxygen bioassays was designed for installation in a van as shown in Figure 2.

The installation was designed around the constant temperature incubator for residual oxygen bioassays, which was the central component of the system. The incubation unit selected was a modified dual voltage refrigerator (Norcold DE727) designed for operation on 115 volt, 60 cycle, single phase or 12 volt D.C. power.

8.2.4.1 *Power Sources* - The 115 volt power system was supplied by an outside weatherproof plug-in. All equipment which required electric power including lights, were wired for 12 volt and 115 volt. The 12 volt D.C. power system was supplied from 2 105 amp 12 volt batteries wired in parallel that could be charged from the 115V power source. A dual battery isolater also provided charging from the vehicle alternator when the engine was running to charge the weakest set of batteries, i.e. the 105 amp system or the single vehicle battery. When both power sources are available, the 115 volt A.C. power was automatically utilized.

8.2.4.2 *Temperature Control System* - A Honeywell T675A single pole double throw thermostat was installed in place of the refrigeration thermostat supplied with the Norcold unit. This Honeywell thermostat was mounted on the temperature control panel with the sensing bulb located inside the back wall of the refrigerator with a capillary tube running from the sensor


40 WATT 115 V LIGHTS
8 WATT 12 V LIGHTS
12 V AIR COMP
115 V AIR COMP
12 V DISCONNECT
TO MICRO FAN MOTOR
TO REFRIG COMP
FUSES
RELAY ISOLATING SWITCHES
TEMP CONTROLLER
CAPILLARY TUBE TO SENSOR
TEMP CONTROL PANEL
INCUBATOR
12 V AIR COMP SWITCH
INCUBATOR CIRCULATING FAN SWITCH
12 V. REG. TERMINALS
115 V. RECEPTICALS
115 V. DISCONNECT
OUTSIDE WEATHERPROOF PLUG IN
115 V. 1 Ø 60 CYCLE POWER SOURCE
12 V 35 WATT HEATING ELEMENT IN INCUBATOR
INVERTER JUNCTION BOX
12 V 105 A BATTERIES
CHARGER 12 V. 15 A
12 V VEHICLE BATTERY
SCHEMATIC ELEVATION OF ELECTRIC WIRING
A INCUBATOR
B FISH HOLDING TANK
C OXYGEN SUPPLY
D BATTERY & CHARGER
E SINK
F TEMPERATURE CONTROL PANEL
G ELECTRICAL CONTROL PANEL
H AIR COMPRESSOR
I STORAGE AREA
PLAN VIEW OF VEHICLE
E.V.S. CONSULTANTS LTD
MOBILE BIOASSY FACILITY
Figure 2

to the thermostat. The incubator was equipped with a circulating fan to provide a rapid temperature control of the samples and incubator bottles, particularly after the unit was opened to remove or replace samples. The fan was operated manually by a pull on, push off switch on the electric power panel. This system provided a temperature control in the incubation unit of ± 0.5°C, well within the requirements of the residual oxygen bioassay procedure.

8.2.4.3 *Aeration System* - Two compressors were provided for aerating samples, one at 115 volt A.C. and one at 12 volt D.C. Each had its own isolating valve which could be opened only when the individual compressor was being used. The discharge from each compressor was tee'd into a 10 valve manifold within the top of the incubator. In addition, an oxygen tank with regulator was also available when oxygenation rather than aeration was desired.

8.3 RESULTS

8.3.1 On Site Screening of Toxic Conditions

On site bioassays were directed at determining initially whether toxic conditions existed at the site, and where they might be located. The working hypothesis of the initial screening test was that for a toxic condition, the residual oxygen value in 100% leachate should be significantly different from control values in dilution water. In order to verify the validity of this procedure as a screening bioassay, the data from 6 field trips was pooled to comprise the field control data of Table 1. Likewise the data in Table 2 represents pooled observations from six sets of laboratory controls. The mean and standard deviation for each of the pooled sets of control data was determined. The means for field and laboratory controls were 2.28 (± 0.18 S.D.) ppm O_2 and 2.22 (± 0.15 S.D.) ppm O_2 respectively. A student *t*-test of significant difference between means showed that with 47° of freedom, the difference between the two controls was not significant at the 5% level. Taking the larger standard deviation (0.18) and multiplying by 1.96 (the 95% confidence interval), a significant difference in residual oxygen values is 0.35 ppm O_2, and this provided the basis for assignment of toxic (T) or non-toxic (N) conditions on site.

The residual oxygen screening bioassays were compared to 96-h assessment bioassays in 100% leachate (Table 3). For samples from site 1, the two procedures showed poor agreement, with the residual oxygen procedure indicating non-toxic conditions in five of six field trips. However, for all other sites (n = 25), agreement was excellent. There were 3 reversals noted, one at site 4, Aug. 6 and site 3, Sept. 3, where the residual oxygen bioassay was more sensitive and one reversal on Aug. 25 at site 4 where the 96-h assessment bioassay was more sensitive than the field residual oxygen bioassay.

8.3.2 Leachate Toxicity Levels and Attenuation

In order to quantify the actual level and attenuation of toxic contaminants, thirty-two 96-h LC50 bioassays were conducted over all sites, and of these, 7 bioassays provided sufficient information to obtain calculations of 96-h LC50 concentrations. Thirty-two determinations of threshold values (TV's) were also made by residual oxygen bioassay in serial dilutions of leachate. Estimated and calculated values for both bioassay procedures are summarized for comparison in Table 3. In all cases the values were in relative agreement, except for leachates from Site 1 for which there was considerable data scatter at higher leachate concentrations. Two 96-h LC50 bioassays were conducted on water samples obtained at Site A above Site 1. These samples had a mean 96-h LC50 value of 30% as shown in Figure 3.

The plot of the mean LC50 values at each site showed a consistent attenuation of

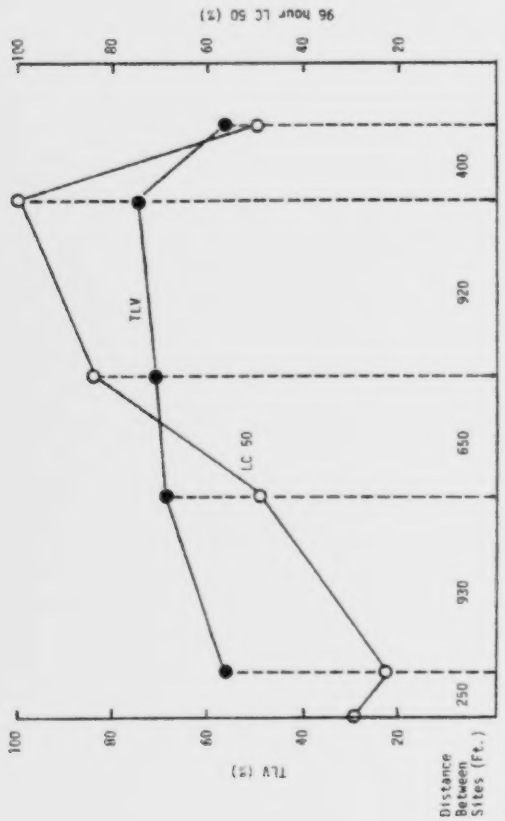


Figure 3. Attenuation of Leachate Toxicity as Determined by 96 hour LC 50 and Threshold Limit Values.

Raw data for the calculation of t values for test of significant difference between field control data (X_1) and laboratory control data (X_2) for Residual Oxygen Bioassay

Table 1. Field Control Data

X_1	$X_1 - \bar{X}_1$	$(X_1 - \bar{X}_1)^2$
2.2	-.08	.0064
2.2	-.01	.0064
2.2	-.08	.0064
2.2	-.08	.0064
2.0	-.28	.0784
2.0	-.28	.0784
2.0	-.28	.0784
2.0	-.28	.0784
2.0	-.28	.0784
2.3	.02	.0004
2.4	.12	.0144
2.5	.22	.0484
2.3	.02	.0004
2.5	.22	.0484
2.2	-.08	.0064
2.4	.12	.0144
2.3	.02	.0004
2.2	-.08	.0064
2.3	.02	.0004
2.3	.02	.0004
2.6	.32	.1024
2.6	.32	.1024
2.3	.02	.0004
2.3	.02	.0004
2.4	.12	.0144
2.5	.22	.0484
59.2		$X_1^2 = .8264$

Table 2. Laboratory Control Data

X_2	$X_2 - \bar{X}_2$	$(X_2 - \bar{X}_2)^2$
2.2	-.02	.0004
2.2	-.02	.0004
2.1	-.12	.0144
2.0	-.22	.0484
2.2	-.02	.0004
2.0	-.22	.0484
2.0	-.22	.0484
2.2	-.02	.0004
2.2	-.02	.0004
2.4	.18	.0324
2.2	-.02	.0004
2.0	-.22	.0484
2.3	.08	.0064
2.2	-.02	.0004
2.3	.08	.0064
2.3	.08	.0064
2.4	.18	.0324
2.5	.28	.0784
2.3	.08	.0064
2.4	.18	.0324
2.4	.18	.0324
2.0	-.22	.0484
2.3	.08	.0064
51.1		$X_2^2 = .4992$

$\bar{X}_1 = 2.28$

$n_1 = 26$

Standard Deviation (S_1) = 0.18

$\bar{X}_2 = 2.22$

$n_2 = 23$

Standard Deviation (S_2) = 0.15

Table 3. Summary Comparison of Toxicity data for Residual Oxygen and Static 96 Hour LC50 Bioassays

Date	Site	96 Hr. LC50 % Leachate est.	96 Hr. LC50 % Leachate calc.	Go - No Go 96 Hr. Bioassays 100%	Go - No Go R.O.B. (Field) 100%	TLV % Leachate est.	TLV % Leachate calc.
Aug. 6/75	1	22	-	T	N*	32	100
	2	25	-	T	T*	45	42
	3	33	29.5	T	T	55	52
	4	100	-	N	T	58	57
	5	35	-	T	T	50	55
Aug. 11/75	1	28	-	T	N	30	31
	2	95	92	T	T	85	100
	3	43	-	T	T	45	46
	4	100	-	N	N	100	100
	5	92	90.8	T	T	79	83
Aug. 18/75	1	21	20.5	T	N	18	17
	2	35	-	T	T	55	71
	3	100	-	N	N	65	70
	4	100	-	N	N	38	40
	5	38	42.5	T	T	44	38
Aug. 21/75	1	25	-	T	N	100	100
	2	45	-	T	T	37	39
	3	100	-	N	N	100	100
	4	100	-	N	N	75	83
	5	59	59	T	T	65	53
Aug. 25/75	1	25	-	T	T	22	32
	2	40	-	T	T	49	57
	3	94	-	T	N	85	85
	4	71	-	T	T	68	69
	5	38	-	T	T	77	69
Sept. 3/75	1	14	15	T	N	100	100
	2	49	-	T	T	100	100
	3	100	-	N	T	100	100
	4	100	-	N	N	100	100
	5	36	-	T	T	42	41

*T = Toxic *N = Non-toxic

toxicity from Site 1, which was the most toxic site, to Site 4 which was often non-toxic. Site 5 was consistently toxic in terms of 96-h LC50 bioassays. Mean values for the residual oxygen bioassays provided a similar, but less accentuated change from site to site. These results were a generally observed pattern throughout the duration of the study.

The controls with 100% leachate and no fish provided an indication of the interference of leachate with high oxygen demand on residual oxygen values. Control samples from Site 1 were consistently depleted of oxygen within the time frame (6-8 hours) of the bioassay. Because of this, the residual oxygen screening bioassays and the threshold value determinations provided unreliable predictions of leachate toxicity at this site. The oxygen demand at this site corresponded to a BOD_5 of 300 ppm (Table 4 and 5). Oxygen demand of samples from the remaining four sites was less than 10% of oxygen values and did not constitute a serious interference. Figure 4 shows the effect of high oxygen demand on a highly toxic leachate and a leachate of low toxicity.

The correlation coefficient (r) of calculated TV's on 96-h LC50 estimates was 0.6 for the equation $Y = a + b \log X$, indicating a significant correlation exists (Figure 5). The data for Site 1 was excluded because of the interference introduced by the high oxygen demand at this site.

8.3.3 Reference Toxicant Biaassays

Reference toxicant bioassays using the residual oxygen bioassay procedure were conducted on three separate occasions, once prior to and twice during the studies. Results are summarized in Table 6. TV's ranged from 60.7 to 72.9 ppb for the reference toxicant sodium pentachlorophenate. It is evident from the data that there is no substantial change in the calculated TV over the 4 month period in spite of the fact that the fish stock tripled in mean weight and the number of fish per unit volume varied from bioassay to bioassay. This indicates that the response of the fish stock over the period of the study was consistent and accordingly, bioassays of leachates may be considered comparable throughout the study.

8.3.4 Chemical Constituents of the Leachates

The results of two sets of chemical analysis of Richmond Landfill leachate are shown in Tables 7 and 8. The overall trend in constituents was to a decrease in concentration from Site No. 1 to Site No. 5, with some exceptions at Site No. 2 which was higher than other sites in zinc, nickel, aluminum and iron. On-site measurements of dissolved oxygen, temperature and pH (Table 5) showed a decrease in dissolved oxygen at Site 5.

8.4 DISCUSSION AND CONCLUSIONS

Chemical analysis of Richmond Landfill leachate showed a larger range in leachate characteristics than obtained by Cameron and Corbett (1975) for the Burns Bog Landfill, although most of the parameters were within a twofold order of concentration. The principal reason for the larger range in data at Richmond Landfill appears due to attenuation of constituents in the drainage ditch.

Toxicity values followed the same pattern of attenuation as chemical parameters for Sites No. 1-4. However, a major anomaly existed with Site No. 5 which showed a substantial increase in toxicity with no comparable increase in dissolved and total iron, sulfide, ammonia, pH or heavy metals. The increase in toxicity is coincident with a decrease in dissolved oxygen at Site 5 (Table 5). An examination of the landfill site (Figure 1) indicates that the toe of the landfill extends through the drainage ditch immediately above Site 5. It is possible that consolidation of the underlying peat has resulted

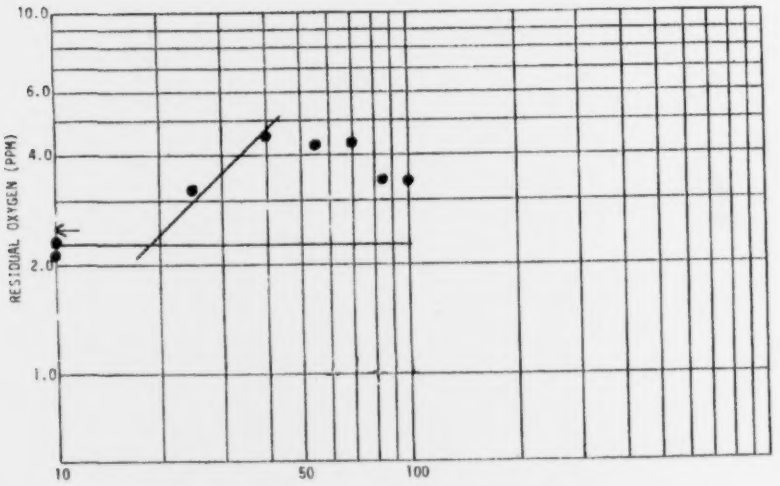


Figure 4A. LEACHATE CONCENTRATION (%V/V)
SITE 1 (18-8-75) TLV (CALC.) = 17%

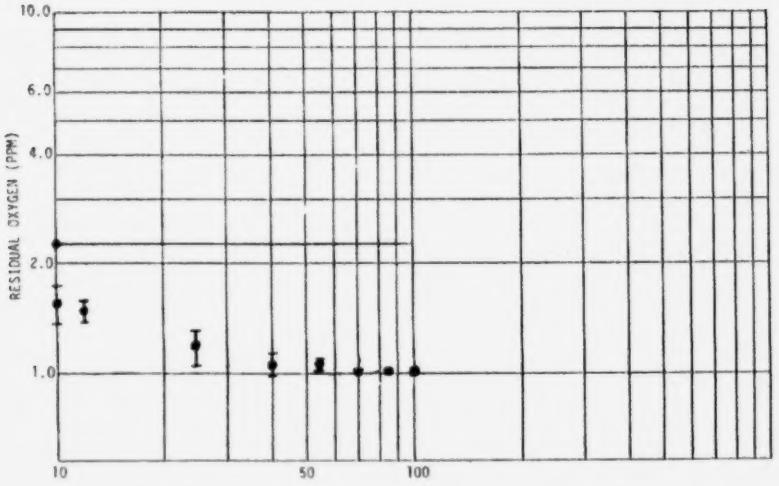


Figure 4B. LEACHATE CONCENTRATION (%V/V)
SITE 1 (3-9-75) TLV (CALC.) = 100%

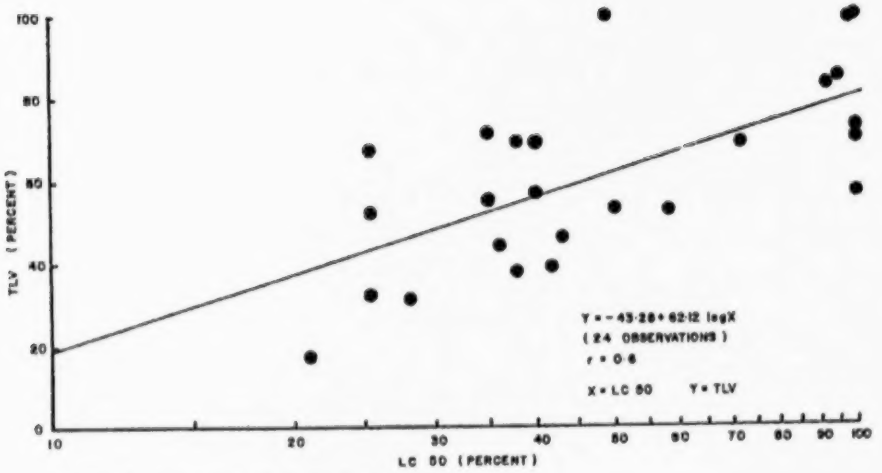


Figure 5. Regression Plot of TLV vs. 96 hour LC 50.

Table 4. Results of Chemical Analysis of Richmond Landfill Leachate at Site #1 (September 3, 1975)

Parameter	Site #1	Units
pH	7.1	-
B.O.D.	300	mg/l
C.O.D.	1100	mg/l
T.O.C.	320	mg/l
Ammonia	32	mg/l N

Table 5. Mean and Range of Results of On Site Measurements of pH, Dissolved Oxygen (D.O.) and Temperature

	pH		D.O.		Temp (^{o}C)	
Site #	Mean	Range*	Mean	Range*	Mean	Range*
1	7.3	6.5-7.8	0.77	0.6-0.8	20.3	17.5-23.5
2	7.4	6.5-7.8	1.9	0.7-3.8	17.4	14.5-21.0
3	7.4	6.6-8.2	4.8	1.6-8.4	17.9	16.5-22.0
4	7.4	7.0-8.1	6.2	3.1-10.8	18.5	17.0-20.0
5	7.2	6.8-8.0	2.3	1.4-3.8	16.8	16.0-18.0

*n = 6

Table 6. Reference Toxicant Test Results Using the Residual Oxygen Bioassay

Toxicant: sodium pentachlorophenate

Test Species: *Salmo gairdneri* Richardson

Test Conditions:

Temp: 10 ± 1.0°C (April 22); 15 ± 1°C (July & August)

pH: 7.0 ± 0.1

Initial Dissolved Oxygen: 9.0 mg/l

Volume: 300 ml

Concentration	Residual Oxygen (mg/l)			
ppb	April 22 3 fish	July 18 2 fish	Aug. 21 1 fish	Aug. 21 2 fish
870	6.9	8.9	8.7	8.3
560	6.0	7.0	6.7	6.3
320	3.6	4.9	5.4	4.2
200	2.2	3.2	3.1	3.0
80	1.2	2.1	2.5	2.5
55	1.0	2.5	2.8	2.7
31.1	1.1	2.1	2.5	2.2
8.7	1.0	2.0	2.4	2.3
Mean fish wt. (g)	0.6	1.76	1.96	1.96
T.L.V. (calc.)	72.9	60.7	63.8	69.9

Table 7. Results of Chemical Analysis of Richmond Landfill Leachate (August 6, 1975)

Parameter	Site #1	Site #2	Site #3	Site #4	Site #5	Units
pH	7.6	7.1	7.6	7.6	6.9	
Alkalinity	1780	1400	975	875	800	mg/l $CaCO_3$
Total Phosphate	3.6	5.3	0.55	0.33	0.48	mg/l P
Sulfide	L0.01	0.2	L0.01	L0.01	0.01	mg/l $S^=$
Ammonia	48	36	22	10	21	mg/l N
Nitrite	L0.005	L0.005	L0.005	L0.005	L0.005	mg/l N
Nitrate	0.01	0.01	1.1	0.6	0.6	mg/l N
Sulphate	26	60	60	50	21	mg/l SO_4
Chloride	350	410	610	820	410	mg/l Cl^-
Conductivity	3800	3400	2900	3600	2500	umhos/cm
B.O.D.	345	123	20	18	13	mg/l
C.O.D.	1800	920	370	300	160	mg/l
Phenol	*	*	*	L0.015	L0.015	mg/l
Non Filterable Residue (NFR)	439	3620	9	67	114	mg/l
Filterable Residue**	2830	2440	2000	2360	1500	mg/l

* interference with analytical peak at 460 nm. However, Samples #1 and #2 both had high apparent phenols, based on color development with reagents.

** calculated, rather than measured directly

Table 8. Results of Analysis of Richmond Landfill Leachates by Atomic Absorption (August 6, 1975)

Parameter	Code*	Site #1	Site #2	Site #3	Site #4	Site #5	Units
Zn	D	1.8	150	0.02	0.04	L0.01	mg/l
	T	5.3	0.7	0.04	0.22	.09	mg/l
Pb	D	0.04	0.04	L0.02	L0.02	L0.02	mg/l
	T	0.13	0.06	L0.02	L0.02	L0.07	mg/l
Cd	D	0.18	L0.01	L0.01	L0.01	L0.01	mg/l
	T	L0.01	L0.01	L0.01	L0.01	L0.01	mg/l
Ni	D	L0.05	0.17	L0.05	L0.05	L0.05	mg/l
	T	L0.05	0.13	L0.05	L0.05	L0.05	mg/l
Cu	D	0.03	0.06	L0.01	L0.01	L0.01	mg/l
	T	0.07	0.06	0.02	0.01	L0.01	mg/l
Cr	D	0.03	0.08	L0.03	L0.03	L0.03	mg/l
	T	0.03	0.06	L0.03	0.04	L0.03	mg/l
Al	D	0.4	28	L0.1	0.6	L0.1	mg/l
	T	1.1	24	0.5	0.4	0.2	mg/l
Fe	D	5.6	56	1.3	7.0	17	mg/l
	T	20	40	1.5	6.3	57	mg/l
Hg		L0.15	L0.15	L0.15	L0.15	L0.15	ug/l
Ca	E	460	250	190	190	200	mg/l
Mg	E	65	87	60	61	51	mg/l
Hardness (mg/l $CaCO_3$)		1400	980	720	730	710	mg/l

*Code E - extractable, unfiltered, preserved by addition of 3 ml/l conc HNO_3 (10 ml/l conc HNO_3 for seawater)

Code D - dissolved, filtered by 0.45 micron membrane filter then preserved by addition of 5 ml/l conc HNO_3 (10 ml/l conc HNO_3 for seawater)

Code T - total unfiltered preserved by addition of 5 ml/l conc HNO_3 (10 ml/l conc HNO_3 for seawater) and acid digested

in the overlying wood mat acting as a wick. The ground water hydrology is such that leachate could move from the active site towards the Fraser River and exit to the drainage ditch through the wood mat "wick". It is possible therefore, that the observed increase in toxicity at Site 5 could arise from an undetected toxic organic leaching from, or through the wood mat into the drainage ditch. Materials such as resin acids and fatty acids are toxic in the 1 to 5 ppm range (Howard and Walden, 1971; Leach and Thakore, 1974) and are toxic constituents of wood waste effluent. An increased organic concentration of 1 to 5 ppm would be within the experimental error of generalized analyses such as BOD_5, COD or TOC and would not necessarily by detected.

This is a clear demonstration that solid waste leachates contain toxic components that are not necessarily identified by routine chemical analysis. Most of the known inorganic contaminants from leachates were analyzed for in this study, but none of the organic contaminants were analyzed for except phenol and those indicated by general parameters such as BOD_5 and COD.

The results of the chemical analyses begs the question; - What is the chemical definition of a leachate? - and emphasizes the value of using toxicity assessment bioassays as an adjunct to ensure the absence of undesirable leachate contaminants entering receiving waters. The unstable character of leachates, and their release from non-point sources further emphasizes the need to utilize on site bioassay procedures to assess their effects on the aquatic environment.

The residual oxygen bioassay procedures described here permit screening bioassays to be conducted at a rate of fifty tests per day on site. Alternatively, some 8 threshold values could be obtained on site per day. These numbers are compatible with the rate of data generation required for broad surveys of surface water contaminants.

Control tests carried out in the field and the laboratory showed no significant difference in the response of test fish in residual oxygen bioassay. This is a finding of major importance inasmuch as a two week acclimation period of fish stocks to new conditions is normally used (Sprague, 1973). In our studies, the lack of such an acclimation period did not result in a significant difference in response.

Threshold values calculated from residual oxygen bioassay data were comparable to and correlated with 96-h LC50 values ($r = 0.6$). Vigers and Maynard (1977) reported almost identical values between the two tests in laboratory bioassays, the residual oxygen values being 0.8 - 1.0 of static 96-h LC50's for bleached kraft mill effluent, chloralkali effluents and sodium pentachlorophenate. McLeay (1976) combined the residual oxygen procedure with elevated temperature stress to obtain elevated residual oxygen values at 0.5 - 1.0 of 96-h LC50 concentrations. Thus, the variance in data is likely due to the unstable nature and oxygen demand of the leachate in an 8-h *vs* 96-h test. It is concluded that a reasonable level of agreement exists between the two tests. The oxygen demand of control samples of leachate caused substantial interference when BOD_5 was in the order of 300 ppm (cf Figure 5 and Table 1). At the next lower BOD_5 tested (123 ppm), residual oxygen threshold values were obtainable.

The mobile bioassay laboratory, specifically designed for application of the residual oxygen bioassay, demonstrated that this new approach to toxicity assessment is reliable sensitive and represents a modest capital expenditure for a support facility. The cost for design and construction of the unit, exclusive of the vehicle was $5900 (1975 dollars). The economies of size are readily apparent when comparing the simplicity of this unit with the required size and design of mobile bioassay laboratories which employ standard bioassay procedures for on-site testing of effluent toxicity (Lake and Loch, 1973; Gerhold, 1973).

Further, the order of magnitude reduction in volumes of test solutions, and time required for bioassay, greatly increases the flexibility for toxicity assessment surveys employing fish. It is concluded that the residual oxygen bioassay adequately assessed the existence and extent of toxicity in leachates from the Richmond Landfill site.

8.5 ACKNOWLEDGEMENTS

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WATCH THE Y IN BIOASSAY

JOHN B. SPRAGUE AND ALVIN FOGELS*

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We need to pay more attention to why we are following certain practices. First of all we should ask ourself why we are doing a bioassay, and then match the procedure to the purpose. This leads to a multiplicity of methods, not to one standard test. A recent tabulation of purposes has been made by an FAO committee and is reviewed. Secondly we should ask why we usually calculate and state fiducial limits of LC50's. They are meant to be used but seldom are. A technique is suggested for telling whether one response is significantly different from another. Thirdly, we should ask why we are standardizing on rainbow trout. Reasons are given for using a small tropical fish for Canadian bioassays.

Sprague, J.B., et A. Fogels. 1977. WATCH THE Y IN BIOASSAY. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5-AR-77-1, Halifax, Canada. pp. 107-118.

Il y a lieu de s'interroger davantage sur certaines pratiques en faisant les essais de toxicité. En premier lieu, demandons-nous pourquoi nous faisons certains essais, pour ensuite juger de la méthode en fonction des objectifs. Cela nous suggérera des procédés variés plutôt qu'un seul essai standardisé. L'étude commente un tableau d'objectifs récemment dressé par un comité de l'OAA. En second lieu, demandons-nous donc pourquoi nous calculons et définissons habituellement les limites de confiance de la concentration léthale. Elles doivent assurément servir, mais si rarement. Une technique suggérée peut indiquer si une certaine réaction diffère sensiblement d'une autre. En troisième lieu, demandons-nous pourquoi nous adoptons la truite arc-en-ciel comme témoin standard. Plusieurs raisons recommanderaient un poisson tropical de petite taille pour les essais biologiques en milieu canadien. Il faut continuellement remettre en question nos méthodes et nos procédés. Des douzaines d'interrogations sur les essais de toxicité viennent à l'esprit mais nous n'en considérons ici que trois.

When we see the word bioassay, it is useful to concentrate on the last letter. It can remind us to continually question why we are following any given procedure. There must be dozens of 'whys' in toxicity testing, but only three are covered here.

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9.1 FIRST Y. WHY IS THE TEST BEING DONE?

This is probably the most important of all possible questions. We should ask ourselves this one at every stage of a testing program. Most of us get so wrapped up in carrying out the procedures of science and technology, that we completely fail to make use of that wonderful tool, the scientific method. Two key items from this method follow, and they must come in order.

- ask yourself the question, "what am I trying to find out?" This should preferably be refined into a question with a yes/no answer.
- design the simplest possible experiment to answer this question.

A group of people recently addressed this general topic. They had been called in by FAO to prepare a report on biological tests for marine pollution. The group decided that the most useful thing they could do, would be to categorize the various types of tests, and why and when they should be used. The report was frankly designed to assist beginners in selecting wisely among the plethora of tests available. Its entire contents cannot be presented here, but a quick look at two of the four lists may be salutary (ACMRR/IABO, 1976). The purposes for doing tests were listed as below.

(1) Screening. For approximate hazardous concentrations, usually with "standard" species and a simple design. A special case would be regulatory tests, rigidly designed on a pass/fail basis, according to a legal standard.

(2) To establish water quality criteria. A wide variety, usually chronic or sublethal, and preferably checked by ecological studies.

The next two are similar.

(3) To monitor effluents. Monitoring and catching peaks of toxicity. Could be on-line tests.

(4) To monitor discharge areas. Surveillance by caged fish and similar means.

The last three form a natural group.

(5) To protect higher trophic levels. Testing whether toxics accumulate and go up the food-web.

(6) To detect tainting or acceptability. Especially to humans.

(7) To detect biostimulation or other indirect effects. Notably eutrophication.

It is not an overly-long list, but obviously there should be quite different designs for tests in each of the categories. Some of the basic types of bioassay procedures are indicated by another of the group's lists, shown below. Explanations are ommitted in most cases.

A. Direct response tests
   1. Toxicity tests
      1.1 Single-sample bioassay, static or constant-flow
      1.2 On-line effluent toxicity bioassay
      1.3 On-line early warning toxicity test
      1.4 In situ toxicity test
   2. Biostimulation

3. Repellence bioassay
4. Bioaccumulation and trophic accumulation
5. Ecological survey

B. Indirect response tests
1. Organoleptic
2. Ecological surveys (e.g. if effect is blanketing by Silt)
3. Biostimulation

There is some repetition from the first list, merely showing that purpose and procedure are tied together.

The group's report then went on to show how the several categorizations fitted together. This became complex; for example, defining good water quality criteria needed most procedures. Finally the group produced a step-by-step analysis of which tests were needed for different types of pollution source.

Perhaps this is all rather obvious and perhaps it is not. The senior author recalls the 1960's when many research workers automatically adopted the "routine" test-procedure of APHA et al. (1965). Hodson has pointed out at this workshop that some of our regulatory tests contain slop-over from research instead of having an efficient pass/fail design using a single concentration. There is some tendency today for a standard all-purpose rainbow trout bioassay. What the ACMRR/IABO report points out is the multiplicity of procedures and reasons for testing, and the numerous combinations of these.

9.2 A SECOND Y. WHY CALCULATE FIDUCIAL LIMITS?

Eight years ago one of us (J.B.S.) was a guest at Pete Doudoroff's university and sat around writing an article about the desirability of calculating "confidence limits" for LC50's and EC50's. Nowadays most of us do this from time to time. The numbers are faithfully set out in one column of the tables in our reports. They give readers an indirect idea of how much the EC50 is likely to slide back and forth along the concentration axis. But most of us don't really use these limits! If we get an LC50 on this week's sample of effluent that is half the value of last week's LC50, we obfuscate our way through a page or so of report, speculating whether this week's result is because of a worse effluent, or because the fish were feeling poorly. Or else we say that this week's effluent is 2.000 times the toxicity of last week's, and completely ignore those fiducial limits sitting in the next column of the table. Why bother calculating limits if we don't use them?

It does not have to be this way! There is a simple way to stop obfuscating and either say that the effluent is definitely worse, or that it is no different, within the accuracy of our test.

This can be done by a standard mathematical procedure called the Standard Error of the Difference. It's not new. It is in statistics texts (e.g. Zar, 1974, pages 105-106). It is tucked in an obscure section of Finney (1971, pages 110-111). It was re-discovered in Sprague's old 1955 notes from graduate pharmacology, and statistics, at Toronto. Finally, a derivation of the formula is in plain view in our old standby Litchfield and Wilcoxon (1949), in a nomographic form.

Basically, the idea is that the Standard Error of the Difference between two means can be derived from the Standard Errors of those two means.

$$S.E._{Diff.} = \sqrt{SE_1^2 + SE_2^2}$$

Why the square and square root? Because you are not allowed to add or subtract S.E., only their squares, in a parallel way that you can't add Standard Deviations, only Variances, which are their squares.

The fiducial limits about LC50's are based on Standard Deviations. There is a predicted relation between S.E. and S.D.

$$SE^2 = \frac{\text{Variance}}{n} \qquad \text{where n is the number of observations}$$

$$\text{or Se} = \frac{SE}{\sqrt{n}}$$

So our formula can become

$$SE_{Diff.} = \sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}$$

If you are a purist, you can go at it this way, then take the difference between the means (LC50's), and do a t-test. However, most of us would probably meet our doom trying to figure out the degrees of freedom.

A more practical way is to use the same procedure as did Litchfield and Wilcoxon, actually a fairly standard one in pharmacology. For one thing you don't have to figure out degrees of freedom and find a table of t. For another thing, to use the "pure" formula with probit analysis, one would really have to weight n according to the distances of the observations from the mean, which is a bit of a job. For a final thing, you can use it on data in the reports of other people who were too ignorant to test differences, even if they merely present LC50 and fiducial limits. The procedure is to take the ratio $\frac{\text{upper fiducial limit of EC50}}{\text{EC50}}$ and do your calculations on that. Litchfield and Wilcoxon call this ratio "f". The upper fiducial limit, being out at the 5% probability level, is really out from the mean at a distance of 1.96 SD. So the answer, the SE_{Diff}, also emerges in a parallel fashion, conveniently at the 5% probability level. It also comes out transformed in terms of EC50. To use the Litchfield/Wilcoxon formula,

$$f_{1,2} = \text{antilog}\sqrt{(\log f_1)^2 + (\log f_2)^2}$$

which I prefer to the equivalent

$$\frac{1.96\ SE_{Diff}}{EC50_{1,2}} = \text{antilog}\sqrt{\left(\log \frac{\text{upper limit}_1}{EC50_1}\right)^2 + \left(\log \frac{\text{upper limit}_2}{EC50_2}\right)^2}$$

Why the logs and antilogs? Because the concentrations are given as arithmetic values, whereas they should be logarithms, the scale of concentrations used in probit analysis. The formula takes only three minutes to work out on any pocket calculator that has logs, square roots, and a memory.

What now? Instead of taking the difference between means (EC50's), take their ratio $\frac{\text{greater EC50}}{\text{lesser EC50}}$

If this ratio is greater than $f_{1,2}$, the two EC50's are significantly different. If the

ratio is smaller, they are the same.

So in our reports, we can stop obfuscating our subjective way through pages of belly-button inspection. Test the LC50's for differences. If not different, say so and forget the weekly ups and downs. If different, look for the reason.

9.3 A THIRD Y. WHY WE SHOULD USE A SMALL TROPICAL FISH

A decade ago some of us were pushing rainbow trout. This section will start by knocking them, in order to end up on a positive note for other fish.

Let's admit that rainbow trout are only fair as a species for laboratory bioassays. Most of us have probably experienced unexplained mass die-offs of a holding tank full of trout. In the basement labs at Guelph, most graduate students working with Salmo gairdneri shut down in January and February, when the fish go "funny" or "touchy" and yield results which are very intersting to explain to a thesis examination committee.

Then there is the problem of aggression among fingerling rainbow trout. This caused endless gnashing of teeth in a couple of recent growth experiments. Mortality had been very low in holding tanks, but as soon as trout were placed in doughnut-shaped growth-tanks with a current, they decided they were in the jungle, set up territories, and started killing each other. One experiment required four starts before trying a size of fish that would tolerate each other. In the other experiment it was necessary to abandon a powerful design which called for cold-branding and measuring growth in individual fish. A change to non-aggressive small fish meant they were too small to brand.

Even routine lethal tests to screen the effluent gave trouble in that same experiment. Over two years, 81 batches of effluent were screened. Many tests were acceptable in general, but there was a tendency for greater mortality in the controls and low concentrations than in high concentrations. Averages could be given but in order to make a point, extreme examples will be used. Some batches of effluent were lethal. Among these, the most peculiar findings were for two sequential batches which had similar results. Combining them gave this strange set of numbers:

% concentration of effluent	0	12.5	25	100
Mortality, 20 fish per concentration	45%	25%	0%	80%

However, most batches of effluent were non-lethal, and the most striking example of the aggression problem was this batch:

% concentration of effluent	0	12.5	25	50	100
Mortality among 10 fish per concentration.	40%	20%	30%	10%	0%

It is a little embarrassing to try to say anything about such results. Correcting for control mortality by Abbott's formula, we would predict that completely replacing a lake with this effluent might benefit the fish with a 67% factor for immortality. The explanation is what was observed in the tanks, that the fish in low concentrations were killing each other, while fish in high but non-lethal concentrations seemed pacified, with no excessive energy for aggression.

Another problem with trout is that they grow during the year and in some seasons one cannot obtain small ones. Tests must go on, regulations may stipulate 50 fish, and statisticians assure us that 30 fish are much to be preferred over 5, and we humbly accept this advice. The problem is that it's difficult to build a sufficiently gigantic diluter in a trailer laboratory, and it's hard to convince the people in Baffin Island to ship down three tank-cars of effluent. The result is that we cram 20 adolescent rainbow trout into a pickle-jar and make do with a 45-gallon drum of effluent.

It has been clearly shown that less than 2 or 3 litres of pulp mill effluent per gram of fish per day gives distorted results in lethal tests (Davis and Mason, 1973). This overloading problem, often encountered with overly-large rainbow trout, is a major one. Many biologists think they get around it by ignoring it, simply because it is "not practical" to build and supply a bioassay system large enough to handle native fish of moderate to large size.

Finally, there is the problem that rainbow trout will not carry out reproduction under the usual laboratory conditions. This is serious, since chronic tests over a reproductive cycle are proving to be one of the most sensitive and meaningful sublethal tests for estimating "safe" levels (Sprague, 1976). Even those native northern fish which will spawn in the laboratory, have a reproductive cycle of a year or two, which makes for an expensive experiment.

Contrast these characteristics with those of small tropical fish which are available for tests, such as flagfish and zebrafish. These are chosen as examples because flagfish are frequently used for chronic tests in the U.S., while we have worked with zebrafish and they will become a standard species for the International Standards Organization.

In a laboratory, flagfish usually remain healthy, seldom die except of old age, and remain non-neurotic in a small aquarium which is almost devoid of natural features. Flagfish and zebrafish are small. They mature and spawn in 2 or 3 months at a size of 2 or 3 cm. This makes for a short and inexpensive chronic test for research purposes. Their fry are much smaller and would be great for testing 20 litres of effluent shipped from Baffin Island. Nor is it any problem to have them reproducing all year in the lab, for a continual supply of small test-fish.

What are the supposed problems in using such species? Firstly these are not native species to Canada. So what? Neither are rainbow trout native to Nova Scotia.

Secondly, you have to test them at a high temperature, unnatural to many parts of Canada for most of the year. This would not seem to be a problem for most pollutants, since we usually test toxicants at the optimum temperature of the test-species. No doubt there should be some checks of toxicity under winter conditions, but this applies also to rainbow trout, usually tested at 15C.

Thirdly, it is widely thought that these tropicals must be more resistant to pollutants than trout. We have investigated this for acute toxicity of several representative materials (Fogels and Sprague, in press). In general, the findings lead to the conclusion that "a fish is a fish". On the average, zebrafish were 2.6 times as

tolerant of the toxicants as rainbow trout, and flagfish were 4.2 times as tolerant. Such differences are negligible. Compare the 5-fold difference in copper toxicity to the same stock of trout in side-by-side labs using the same water and similar apparatus. (Fogels and Sprague, in press; Howarth, 1976). Compare the 2.5-fold fluctuation in effects of several toxicants to the same stock of rainbow trout in a British lab (Brown, 1968). Compare results in different laboratories giving a range of 8.6 times for toxicity of copper to rainbow trout in similar water (Goettl et al., 1973; Calamari and Marchetti, 1973). Even a carefully controlled interlaboratory standardization resulted in a 2-fold variation of response of trout to pentachlorophenate (Davis and Hoos, 1975). The list is extensive (Sprague and Fogels, in press; Black et al., 1976). We must almost expect unexplained variation up to an order of magnitude in tests of acute lethality.

The only difficulty with using a slightly more tolerant tropical fish would be for testing effluents which are marginally lethal at full strength. In such cases, a sensitive organism would be desirable, and indeed, something such as Daphnia would probably be more desirable than trout.

Sublethal testing is perhaps of more significance in tests to protect our waters, and some experiments have shown that flagfish may be equally sensitive as trout. A recent paper says that "the lowest cadmium and zinc concentrations causing adverse effects to the flagfish were similar to those affecting" brook trout and rainbow trout (Spehar, 1976). Tests of a whole effluent yielded similar conclusions (Rowe et al., in preparation). When flagfish were exposed to the effluent, growth was more sensitive than reproduction, and the threshold for effect was very close to 10% effluent. This affected males of the first generation but not females and not the second generation. For trout, the threshold for effects on growth was again very close to 10%, which caused a decrease in only the fourth of four growth periods at one feeding rate, and similar marginal effect at another feeding rate.

The final question may be the common reaction "Who cares if a pollutant kills a zebrafish? Can I catch it in the Schubenacadie river?" The only answer to this is education to wear down such prejudice, along the lines of that argument "fish are fish", which may not be too far from the truth as far as pollution is concerned.

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9.5 QUESTION PERIOD

Jerry Payne: With the two hundred mile limit now coming into effect, most of our waters will be salt. I'm wondering if you have any ideas for good marine species that we could possibly use?

John Sprague: The learned FAO committee did not come up with a satisfactory answer to that, and I can't either. When working at St. Andrews, I used small winter flounders, but these are not ideal. One present solution is to use rainbow trout acclimated to sea-water, and that should be okay. Another alternative would be the use of lobster larvae, which are sensitive, or some other invertebrate.

Cecil Innis: While I agree with your basic thesis, John, since we have both species in our laboratory, I can't help feeling that the differences that show up in continuous-flow are probably differences due to the variability of dosing systems. That appears to me to be one of the biggest problems in making the continuous-flow bioassay a viable tool. I can't help feeling that when working with something as toxic as copper, a malfunction occurring at 2 minutes past twelve on Sunday night may be missed completely. That is the end of the test as far as its viability goes. I once watched an experiment for about two hours and no toxicant was delivered by the dilutor. If that had been a 4-day experiment the results might have been rather strange. I suspect that much of the variability that we're talking about with the continuous-flow is probably an artifact of the dosing system.

John Sprague: No question that this could be one problem. A solution would be to have a sufficient volume of test-water, as well as a satisfactory turnover time, in order to tide the experiment over short periods of erratic behaviour. However that again leads to the problem of larger capacities.

Bob Cook: What about using a tropical marine fish? If indeed a "fish is a fish" and we don't have to consider a specific species, following your line of thought, you could get a tropical marine fish which will very rapidly meet all the criteria that you specify.

John Sprague: Since you are using my argument, I wouldn't raise any objection, in fact I think we may come to it. Phil Butler from Florida was on that FAO committee, and suggested a southern marine killifish, although he did not regard it as completely satisfactory.

Rick Hoos: If a "fish is a fish", why worry about whether it is salt water or fresh water? It depends on your first Y though, what your objective is.

John Sprague: Yes that's a logical projection of the argument. I've always felt the difference in sensitivity was not that great between marine and freshwater fish. We tested them side-by-side at St. Andrews, and in fact, during the Arrow oil spill, checked out dispersants for marine use, both with lobsters and with salmon in freshwater. The only

thing to watch for might be special behaviour of some chemicals in sea water, and a chemist might be able to predict that.

Ed Pessah: We found some major differences in D.S.S. values. Do I detect some measure of trying to get another sort of standard fish, John?

John Sprague: Not really, although there is an advantage in piling up complementary information on one species. The main things in my mind were the advantages of having smaller fish, and ones that reproduce quickly, so that chronic and acute tests could be tied together for the same species.

Blaine Hummel: Have you tried minnows or sticklebacks or small cold water fish?

John Sprague: Not personally. Of course fathead minnows are used a lot, but they still have a relatively long reproductive cycle.

Gary Vigers: I don't really see the necessity for conducting lethal bioassays on the same species that you want to conduct sublethal work on. What's the rationale? I don't see any connection.

John Sprague: I can't defend that very well having said that "a fish is a fish". The rationale would be that one is usually looking for an application factor.

Gary Vigers: But you can apply that between species as easily.

John Sprague: Yes, you can, but I must admit that in many people's minds, one source of possible error would be removed by using the same species for both acute and sublethal work.

Howard McCormick: While thinking of changing to a tropical species, I have intuitive concern that if you were to select the guppy, that was popular some time ago, you'd get into a different reproductive mode and if you're going to do chronic life history studies, you may not get the same type of response to a live born larva as opposed to one that passes through an egg period. That's an intuitive feeling on my part.

One advantage of a flagfish that Dr. Sprague did not mention directly but implied is the time involved in running a chronic study. I know the interest here today and yesterday has been largely with acute studies, but in chronic tests with this warm temperature of 25°C and that particular species, the flagfish can be taken from egg to reproducing adult in eight weeks time. This is quite an advantage too.

John Sprague: You are correct on both points as far as I am concerned. The guppy seems to have some other peculiarities as well as its live-bearing.

Gordon Craig: To qualify the remark made by Mr. McCormick, the flagfish is easy to breed and can be cultured through a life cycle in a short period. However there are limiting factors. The time required to complete a life cycle is inversely related to the attention and feeding received by the fish. Secondly the effect of residual chlorines in dechlorinated laboratory water supplies has a retarding effect on the reproductive response. Best results are obtained using sources of natural water.

Peter Hodson: I'd like to take you back to rainbow trout. One problem with rainbow trout, is that when testing them with lethal or near-lethal concentrations of heavy metals (cadmium, lead, copper) they are very hypersensitive. When you walk by the tank they suddenly go berserk, "roar" around the tank and a significant number will roll over. If you check your fish and notice that one fish is looking moribund, you'll come back a few minutes later to recheck him and the chances are that there'll be two moribund fish. A

few minutes later a recheck shows three fish looking moribund. This tends to very nicely influence our results - we could pick any LC50 we wanted and check frequently enough until all the fish die. I've only seen this with rainbow trout. We may also have a bioassay for the effect of toxicant on aggression. Obviously aggression is natural and if aggression decreases at a specific toxicant concentration, and it is a significant effect, then we should use it as a bioassay. Instead of bemoaning this property of rainbow trout, we can take advantage of it.

John Sprague: Stories circulate about fish being sensitive to disturbance when tested against insecticides. Clarence Tarzwell tells about knocking on the side of the test-tank and getting a large number of fish rolling over, when testing an insecticide that had been spilled into the Mississippi. I think he was using a clupeid.

Rick Hoos: Going back to John's apparent hang-up over loading density which he emphasized-again, if we're that worried about loading density, we should be considering other, smaller species that may be totally different from fish, such as algae or perhaps some invertebrate forms. But the basic question that was raised at the beginning is why do you do the test? Depending on whether the objectives are regulatory or monitoring, or whether one is attempting to relate to the Canadian scene or the Canadian environment, or whether one is trying to carry out a longer term chronic test -- all have different objectives, and may necessitate use of different test species. That's really what it comes down to.

John Sprague: Yes, I agree. One of my current favourites in Daphnia, although there is an art in culturing them. Gary Westlake got very good results from two-week reproductive tests with Daphnia pulex, when we were working on a refinery effluent.

Don McLeay: I certainly can see the many virtues of using tropical fish. It seems to be the only economical way to cope with some of these long-term studies and I also don't like rainbow trout, but I think that the wide variability in the toxicity data reported in the literature for rainbow trout with a given toxicant is a result of the number of poor tests that have been done or the diversified test conditions, rather than the actual innate variability in tolerance of this species. The other point I wanted to raise was in situations where we're testing complex industrial wastes such as pulpmill effluents where we're trying to protect coldwater species such as salmon, I think the toxicity of these effluents is going to be decreased appreciably at say 25°C as a test temperature rather than at a colder temperature. You tend to get an effective waste treatment system operating at this temperature during the test which will reduce the toxicity. And also I'm a little concerned that, as you indicated, trout are considerably more sensitive than flagfish. Will the use of flagfish really offer good protection for resident salmonid species in all instances or do we have to come back to application factors to give a good measure of protection?

John Sprague: The waste treatment phenomenon could be serious - I hadn't thought of it much. Good turnover times might help and thus would be easier with small fish. As to trout being "considerably more sensitive", the thesis of Alvis Fogels was that the difference is not an important one, looking at the overall picture. No doubt we should use all information at our disposal, including application factors.

Gary Westlake: I am concerned that the statistical test you suggested might lead to comparisons between unlike observations. To take the absurd extreme, one might compare fish with elephants. Do we not have to make the assumption that the observations come from the same population?

John Sprague: I'll try to resist any inane comment about elephants being only semi-aquatic. Gary, you are a better statistician than I, so no doubt we should be a little careful. Personally I tend to take the naive approach that any standard deviation is a measure of scatter in a particular test and can be compared with any other. If this leads to the conclusion that two results are different, I'd accept it until somebody stopped me. Of course, one should be careful to examine all possible reasons for the difference, and not credit it to a 'favourite' reason without some thought.

John Davis: I rather appreciate your approach that a fish is a fish and I wonder if you'd make the jump to saying that zebrafish or flagfish are perfectly adequate for defining water quality criteria. If you take the range of variability in establishing incipient toxic or incipient sublethal levels and you use the most sensitive end of that range, do you think that would approximate a safe water quality criteria number? Do you think that would cover the kind of concern that Don expressed about the sensitive salmonids and that sort of thing?

John Sprague: Yes, I think so. Especially as a first approximation. In important situations, it is highly desirable to do what you did in B.C., take your machinery out to the real world and do field-testing. That is the final answer.

Bob Cook: John, following on that suggestion then you use zebrafish and flagfish to get your fish number, then you could use Daphnia and some other invertebrate that doesn't relate to the Canadian scene to get your invertebrate number. You could even use a South African algae that might be handy to rear. It seems that our criteria for the Canadian environment might not relate to what the Canadian environment really needs.

John Sprague: It might not, but Fogels and I are saying that the weight of evidence is the other way. Again, we need to shorten and simplify our primary lab-testing so we have time to get out and check it with actual or simulated field-testing.

Gary Vigers: Looking at some of the numbers on sensitivity, you mentioned flagfish is 4.2 times as sensitive as rainbow trout. In some recent work that we've done, we've found that herring are about 5 times as sensitive as rainbow trout. That is pretty close to a factor of 20 times for lethal effects. If there are other species in the Canadian environment - sand smelt, sand lance, etc. - that are even more sensitive and this is quite feasible, then I suggest that we're outside of the factor of 10 that you were suggesting earlier as being a reasonable limit. We're no longer relating then to the Canadian situation, and that has to be one of the criteria, regardless.

John Sprague: Well a 20-times factor is certainly appreciable. One of the by-words in the U.S. was testing "the most senstive local species". Maybe that would be a second stage, and actual or simulated ecological studies would be the final step.

Ed Pessah: You started by saying that for various reasons, for regulatory purposes, it's fine to standardize; then you said that for developing environmental quality standards, for doing a host of research applications, one ought not to standardize. Now you're saying that a fish is a fish is a fish, so why not standardize, get a lot of information on one fish, understand the biology of that organism and then start extrapolating that information to a whole series of other organisms. Let's take it a little further. Should we standardize on a select group of various organisms and research extensively on them to get the biological information. You can standardize in various places, in your approach - in your test species, in your pre-test conditions. It appears to me that in some of these areas there would be great advantages in standardizing. Are you suggesting that we in

fact start working as a group, working on three species, two invertebrate species and a couple of algal species, in the hopes of getting together generalities more quickly in the field of aquatic toxicology and in environmental biology as a whole?

John Sprague: Yes, I would agree with that. At the same time, one should not feel locked in to some sacred species if there is good reason for using another one. I'll give a minor apology for saying different things at different times.

Gordon Craig: Regarding John Davis' question about how representative are flagfish responses of indigenous species - would water quality criteria be based on tropical fish responses? We have observed flagfish reproductive responses in the presence of depressed pH and copper sulphate. Both toxicants produced thresholds at levels which compared favorably with those reported for fathead minnows and brook trout.

Cecil Innis: The question really is this - what works? Rainbow trout bioassays work, flagfish bioassays work. I keep on hearing people saying that smaller is better and more sensitive. Last year I ran three continuous-flow experiments with rainbow trout and Gammarus pseudolimneaus living in the same tanks, and the rainbow trout died at concentration levels three times lower than the Gammarus. We have had all kinds of trouble with Gammarus pseudolimneaus and we virtually have abandoned it as a test animal. If people do not wish to use flagfish or rainbow trout and they wish to use something else, they should show us how it works. If we wish to protect the Canadian environment with Canadian fish, then someone should define a Canadian fish that we can use and do inexpensive life cycle studies in the laboratory with it.

Ron Wallace: I'd not like to see Gammarus pseudolimneaus adopted as a standard because we'd kill a lot of a little creature that I'm pretty fond of, but I think it is technically feasible if people were really interested in it.

I wanted to direct a comment towards John Sprague. John, a fish might be a fish, but in cases of whole effluent screening or in regulatory tests or even in research tests, is an effluent always an effluent? That is to say, have you checked in terms of a temperature sensitive chemical species - the volatility effects or recombination effects or chemical interactions at the higher temperatures - to see if the flagfish versus the rainbow trout relationship is holding true. I'm just wondering if at the higher temperatures you're going to be generating some chemical spinoff effects that you wouldn't be getting at the comparatively lower temperatures that you'd be running with the trout.

John Sprague: As far as the fish themselves are concerned, I don't see a problem with temperature or similar factors. There's no particular reason why a flagfish at its warm optimum should be different from a trout at its moderate optimum. Even for a trout, one would find it impossible to predict whether a pollutant would become more or less toxic at higher temperature. But what you are driving at is a real danger - actual changes in the toxicant depending on the modifying factors. We should ask chemists about things like that, and listen carefully.

Gloria Sangalang: Again, this is just going back to the "fish is a fish" statement. Most of our bioassays have considered the influence of temperature, light and various other things on the effect of toxicants on fish, but many investigators have in fact disregarded the fish itself as an experimental animal. By this I mean, are the fish they are using in the same state of sexual maturity, are they the same sexes, etc., etc. In our experience we have found out that fish in one state of maturity are more sensitive to a particular toxicant than then the same kind of fish in a different state of maturity and this is one important point that many investigators have overlooked.

AN EVALUATION OF DEATH BY HYPOXIA IN A MARINE FISH AS AN INDICATOR OF OIL DISPERSANT TOXICITY

JOSEPH W. KICENIUK AND JEREMIAH F. PAYNE*

Kiceniuk, J.W. and J.F. Payne , 1977. AN EVALUATION OF DEATH BY HYPOXIA IN A MARINE FISH AS AN INDICATOR OF OIL DISPERSANT TOXICITY. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., November 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5AR-77-1, Halifax, Canada, pp. 119-122.

There may be a correlation between the toxicity of a substance and oxygen tension after death by hypoxia. We have investigated this hypothesis using a series of commercial oil spill dispersants. Most dispersants even at concentrations of 2000-2500 ppm did not change the oxygen tension at death of a pelagic marine fish, Mallotus villosus. There appeared to be a trend towards higher residual oxygen tensions with alkylphenol polyethoxylate type detergents (known to be highly toxic). This trend is evident at much higher concentrations (-30 times) than the bradycardia threshold for this species. Oxygen tension after death by hypoxia may not be a reliable sensitive indicator for general use in toxicity studies. Possible sites of action of nonionic detergents are discussed.

Kiceniuk, J.W. and J.F. Payne, 1977. AN EVALUATION OF DEATH BY HYPOXIA IN A MARINE FISH AS AN INDICATOR OF OIL DISPERSANT TOXICITY. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., November 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5AR-77-1, Halifax, Canada, pp. 119-122.

D'aucuns ont suggéré qu'il peut y avoir un lien entre la toxicité et la tension oxygénique résultant du décès par hypoxie. Nous avons mis cette hypothèse à l'épreuve en utilisant une série de produits dispersifs commerciaux habituellement appliqués aux déversements de pétrole. La plupart des produits dispersifs, même à des concentrations de 2000 à 2500 ppm, n'ont pas modifié la tension oxygénique post mortem d'un poisson pélagique, le Mallotus villosus. Nous avons cru observer une tendance vers des tensions supérieures d'oxygène résiduel à l'emploi de détergents du type alkylphenol polyethoxylate, qui ont la réputation d'être extrêmement toxiques. Cette tendance devient évidente à des concentrations beaucoup plus élévees (- 30 fois) que le seuil de bradycardie de cette espèce. Il se peut que la tension oxygénique post mortem ne soit pas un indicateur suffisamment sensible et fiable pour servir généralement aux épreuves de toxicité. Nous commentons certains champs d'application des détergents non ioniques.

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10.1 INTRODUCTION

Many methods have been developed to measure the toxicity of various pollutants on fish (Sprague 1969, 1970, 1971). A method which is attractive because of its simplicity is the residual oxygen method (Carter 1962). A combination of toxicant plus oxygen depletion causes a faster kill than toxicant alone and is therefore quicker to use than conventional lethal toxicity tests. Carter (1962) found this method useful with compounds which killed fish in less than 96 hours. Oil dispersants cause bradycardia in fish (Kiceniuk et al. 1976), similar to the effect of hypoxia (Holeton and Randall 1967; Butler and Taylor 1975). Therefore, the application of Carter's method to the study of dispersant toxicity could provide information on the utility of the method for dispersants. Some information may also be obtained on their site and mode of action.

10.2 METHODS AND MATERIALS

Corexit 7664 and 8666 are manufactured by Esso Chemicals Canada Ltd., Duosol and Actusol by DuBois Chemicals of Canada, Synperonic O.S.D. 20 by Canadian Industries Ltd., BP1100X by BP Trading Ltd. and Oilsperse 43 by Diachem of B.C. Ltd. Other chemicals were obtained from British Drug House Chemicals Ltd. or Sigma.

10.2.1 Specimens

Male capelin (Mallotus villosus) were caught on a local beach in July during the spawn and were held in cylindrical 4000 l tanks at ambient seawater temperature. The fish remained in good condition throughout the experimental period and were not fed.

Concentrations are expressed as volume dilutions in seawater. Test emulsions were prepared by adding the required volume of dispersant to seawater, diluting to 4 l, and mixing by aeration. No attempt was made to measure soluble or accommodated levels; a meaningful concentration expression in terms of soluble or accommodated levels is difficult to determine since the varying sized globules which were initially present upon mixing formed 'surface' slicks over the assay period. One fish (25-35g) was placed in a 900 ml glass jar filled with an air-equilibrated test solution. Each jar was filled to eliminate air bubbles, and the lid secured. Controls were done the same except that clean aerated seawater was used as the test solution. After death of the animals (~ 1 h) water samples were taken with a syringe and the oxygen tensions determined with a Radiometer electrode type E5046.

10.3 RESULTS

Residual oxygen tensions at fish death did not differ significantly from control values (Fig. 1). Compounds such as Triton X100, Actusol and Duosol, known to be toxic to fish, showed a trend towards higher residual oxygen tension. No such trend was evident with the less toxic dispersants as Corexit 8666 and 7664, BP1100X or Oilsperse 43. There appeared to be no significant difference in response from fish kept for 1-2 weeks or 1-2 days. The concentrations of some test materials required to produce detectable bradycardia in capelin (Kiceniuk, unpublished) are indicated in Fig. 1 for comparison.

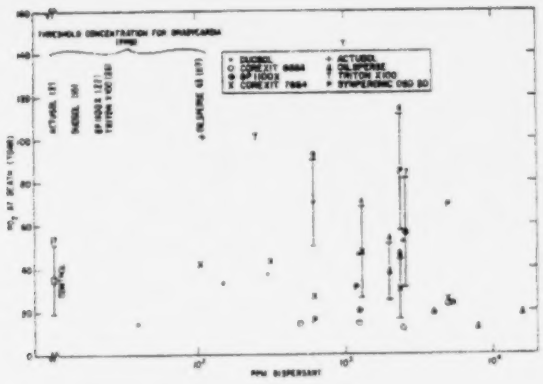

Fig. 1. Residual water oxygen at fish death in the presence of varying concentrations of dispersants. Vertical lines represent ± one standard deviation of the mean of n trials (indicated atop vertical lines). Points with no statistics represent single trials.

10.4 DISCUSSION

To provide a measure of the toxicity of a material, the residual oxygen tension upon death due to a combination of toxicant and hypoxia must be different from the residual oxygen tension after death by hypoxia alone. The measurement of residual oxygen tension upon death (due to a combination of hypoxia and oil dispersants) does not meet the above criteria and therefore cannot give an indication of the acute toxicity of the dispersant. The residual oxygen can be expected to increase only if toxicity follows one of the following general modes of action:

1. Interference with carbon dioxide unloading at the gills resulting in a reduction of blood oxygen loading.
2. Interference with uptake of oxygen at the gills resulting in a fall in the amount of oxygen available to the tissues.
3. Interference with oxygen transport to the brain resulting in brain death at a higher environmental PO_2 than normal.
4. Blockage of components of aerobic metabolic pathways would cause death by cytotoxic hypoxia at higher residual PO_2 than in controls.

The cardiovascular system is capable of considerable compensations in the transport of oxygen to tissues by changes in cardiac output and arterio venous oxygen difference (Butler and Taylor, 1975; Kiceniuk and Jones, 1977). This compensation would be expected to buffer, to some extent, toxicity by modes 1, 2 and 3. Toxicity by mode 4, on the otherhand, would be expected to have a more dramatic effect on the residual oxygen tension since no compensation is known to exist. Cyanide, one of the materials the residual oxygen method was first used for (Carter, 1962), is a known cytotoxic compound (Swinyard, 1975).

Low concentrations of all the dispersants tested resulted in bradycardia (Kiceniuk, unpublished) indicating that the cardiovascular system is involved in dispersant toxicity. Measurements of arterial blood oxygen content and partial pressure in another marine species (Tautogolabrus adspersus) have shown that the arterial blood continues to be well oxygenated during exposure of the fish to dispersant (Kiceniuk, unpublished) indicating

that there is no interference with oxygen uptake or loading of the blood (modes 1 and 2). We therefore suggest that the probable action of dispersants is on the transport system (mode 3).

10.5 ACKNOWLEDGMENTS

We thank E. Pessah, Environmental Protection Service, Dartmouth, Nova Scotia for dispersant samples: Marine Sciences Research Laboratory, Memorial University for experimental facilities and W. R. Squires for field assistance.

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10.7 QUESTION PERIOD

Gary Vigers: Comparing the last graph with the table you showed earlier, did that have all the temperatures that you showed in your original work?

Jerry Payne: No, the residual oxygen work with capelin was carried out at 10-12 C only.

John Davis: I think the residual oxygen test may be somewhat useful for approximating an acutely toxic level and with some substance down into the sublethal but it is not likely to usefully predict a sublethal threshold.

Jerry Payne: I think the work with dispersants supports this hypothesis.

Don McLeay: I know some work has been done with marine fish where the residual oxygen test has worked. Possibly you were hitting the fish with too heavy a hammer if they were dying within an hour. We find that for Salmonid species, fish loading and test temperatures must be balanced so that fish survive in this test for 4 to 5 hours if a sensitive measure of toxicity is to be obtained.

Jerry Payne: Possibly, it is certainly worth trying to see if sensitivity is increased if fish are allowed to survive for 4 to 5 hours or longer.

CHANGES IN STEROID HORMONE METABOLISM AS A SENSITIVE METHOD OF MONITORING POLLUTANTS AND CONTAMINANTS

HARRY C. FREEMAN AND GLORIA SANGALANG*

Freeman, H.C., and G. Sangalang. 1977. CHANGES IN STEROID HORMONE METABOLISM AS A SENSITIVE METHOD OF MONITORING POLLUTANTS AND CONTAMINANTS. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5-AR-77-1, Halifax, Canada. pp. 123-132.

Fish, known to be sensitive to sublethal levels of pollutants, are used as sensitive indicators to determine the presence of these compounds in water. Methods are given to determine the effects of trace quantities of contaminants or pollutants on steroidogenesis. Examples and data are given of altered steroid hormone metabolism in brook trout and also a marine mammal, the seal.

Freeman, H.C., and G. Sangalang. 1977. CHANGES IN STEROID HORMONE METABOLISM AS A SENSITIVE METHOD OF MONITORING POLLUTANTS AND CONTAMINANTS. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5-AR-77-1, Halifax, Canada. pp. 123-132.

Les poissons, connus pour être sensibles au niveau sous-mortel des polluants, sont employés comme indicateurs sensibles pour indiquer la présence de ces substances dans l'eau. Des méthodes sont données pour montrer les effets de quantites en traces de contaminants ou de polluants sur le steroidogenesis. Des examples et des datas sont donnés concernant des stéroides qui sont altéres dans le metabolisme des hormones chez le truite et aussi chez le phoque, mammaire marin.

11.1 INTRODUCTION

Monitoring the effects of pollutants in order to have an early warning of a pollution problem or checking the toxicity of a potential pollutant is a difficult task using methods that are presently available. It is obvious that there will be problems with synergism and antagonism due to the complex nature of many pollutants. It is desirable to have methods sensitive enough to warn of a possible problem before it becomes so serious that it is irreversible. One method would be to check the local flora and fauna of the area in question for any deleterious or toxicological effects of suspected pollutants. This would necessitate knowing the natural state and condition of the species in the area of concern in order to detect any changes.

It is known that in order for a pollutant to eliminate a species, it is not necessary for it to be lethal. A species could be eliminated if its reproductive capacity is lowered below a critical level or if it is incapable of reproducing.

Fish have been shown to be sensitive to low levels of pollutants (Pickering and

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Henderson, 1966; Gardner and Yevich, 1970; Eisler, 1971; Sangalang and O'Halloran, 1973; Sangalang and Freeman, 1975; Freeman and Idler, 1975; and Freeman *et al.*, 1975) and may be used as sensitive indicators for an early warning of pollution in either fresh or salt water. The steroid hormones present and pathways of synthesis are known for several species of fish (Idler, 1972). Sangalang and O'Halloran, 1973; and Freeman and Idler, 1975, have shown that sub-lethal levels of certain contaminants have altered steroidogenesis in fish and have also significantly altered their reproductive capacity. The effects of trace quantities on sub-lethal levels of pollutants or contaminants may be determined by comparing steroidogenesis of exposed fish with those of unexposed fish of approximately the same size, age, sex, and state of maturation. Changes in their steroid hormone metabolism give some insight into changes in the normal physiological function of the fish, in the case of mineralo-corticosteroids, osmoregulation; in the case of glucocorticosteroids, stress capabilities; and in the case of androgens and estrogens, reproduction.

The purpose of the present report is to demonstrate the sensitivity of the steroid hormone metabolism method in detecting the effects of sublethal levels of contaminants in fish and describe procedures by which the altered steroid hormone metabolism (*in vivo* or *in vitro*) by the gonads and/or the interrenals in fish may be determined.

11.2 MATERIALS AND METHODS

11.2.1 Experimental Fish

Fish, in each group, for experimentation should be normal fish of the same species, approximately the same size, sex, age and state of maturation. Control and treated fish groups must be held under exactly the same conditions with the only difference being the water containing the pollutant under test or the food containing the pollutant.

11.2.2 *In Vitro* Steroidogenesis Tests

At the time of sacrifice, the gonads and/or the interrenals (adrenal homologues in fish) from each group of animals are immediately excised. The organs freed from extraneous tissue, are kept in ice-cold incubation medium under oxygen for a short time until incubated or frozen immediately on dry ice until used. A Tris-buffered incubation medium as described by Idler and Truscott, 1966 is used. Immediately before incubation, glands from each group are minced and mixed thoroughly to form homogeneous interrenal and gonad tissue pools. A one gram sample of each tissue pool and 10 ml of incubation medium are used for each incubation experiment. All incubations are carried out simultaneously under an atmosphere of oxygen in a constant temperature shaking incubator. All incubations are carried out at the normal water temperature of the fish as this is important in order to maintain normal enzyme activities.

In each experiment, the tissue is pre-incubated under oxygen in 10 ml of the buffer containing a NADPH-generating system as described by Idler and Truscott, 1966 for ½ hr. to reduce the endogenous pool of steroid substrates. The tissues are then incubated in 10 ml of fresh buffer containing fresh co-factors and equimolar amounts (ca. 0.025μmoles is suitable) of radioactive steroid substrates [^{3}H]-pregnenolone (ca. 7.5μCi) and [4-^{14}C]-progesterone (ca. 1.4μCi) for 4 hr. The precursors are added to the medium in 0.1 ml of propylene glycol. Tissue samples from each pool are also incubated for 4 hr, in the same manner but without radioactive substrates. These samples later serve for the determination of recoveries using added tracers when analyzed by double isotope derivative assay (DIDA) or radio immunoassay (RIA) procedures to check the quantity of steroid products, thereby confirming the autoradiography results. At the end of 4 hr., the

incubations are stopped by the addition of acetone or by freezing on dry ice. The incubations may be stored at ca. -40°C until analysis.

11.2.3 Extraction of Metabolites

Before solvent extraction ca. 30-40 µg each of unlabelled steroids, similar to the ones expected in the species under investigation, are added as carriers (Sangalang and Freeman, 1974; and Freeman and Idler, 1975). Carrier steroids and corresponding [^{14}C]-labelled tracer steroids ca. 15,000 d.p.m. of each are added to the incubations without radioactive precursors to determine recoveries through the isolation procedures. The interrenal and testicular incubations are extracted with 8 volumes of dichloromethane and partitioned with aqueous solvents as described earlier by Freeman and Sangalang, 1976. The estrogens and neutral steroids are extracted from the ovary incubations by an ethanol-acetone extraction method followed by phenolic partitioning as reported by Freeman *et al.*, 1975.

One-tenth of each crude extract is spotted on silica gel plates (Merck GF254 for androgens and adrenocorticosteroids and Merck HF254+366 for estrogens and neutral steroids). The plates are then developed in appropriate solvent systems as described by Freeman *et al.*, 1975 and Freeman and Sangalang, 1977.

11.2.4 Autoradiography

The chromatoplates are exposed for 48 hr. to a no-screen medical x-ray film (Kodak Blue brand). The exposed x-ray film is developed according to the manufacturer's specifications. The locations of the radioactive spots, as seen on the autoradiogram, are compared with the locations of the ultra-violet fluorescence quenching carrier steroids. Any alterations in steroid hormone metabolism are clearly indicated by comparing the autoradiograms of the controls with those from the contaminated fish.

The radioactive chromatograms may also be observed in an instrument equipped with a radiochromatogram imaging system (e.g. Beta Graph, Panax). Radioactive areas are photographed with a built-in polaroid camera that permits instant viewing of the radiograms with little handling. This instrument rapidly detects both ^{3}H and ^{14}C active metabolites but is not as sensitive as "x-ray" autoradiography.

11.2.5 Tests for Effects of Pollutants and Contaminants

11.2.5.1 *In Vitro* Tests

The steroid hormone biosynthesis of at least 5 fish of approximately the same age, size, sex and state of maturation from a suspected polluted or contaminated area are compared with those from 5 or more fish from a non-polluted area. This is done by removing the gonads and/or interrenals from both groups and incubating them following the procedure described in the *in vitro* steroidogenesis tests followed by the extraction of the metabolites. The products formed, quantities synthesized, and pathways of synthesis are estimated quickly by autoradiography. The autoradiography results may be confirmed by radioimmunoassay (RIA) (Tyler *et al.*, 1973; and Abraham, 1974, 1975), or by double isotope derivative assay (DIDA) procedures (Sangalang and O'Halloran, 1973; Freeman *et al.*, 1975; and Freeman and Sangalang, 1976). The RIA assay procedures may be carried out quickly and accurately but the DIDA procedures are lengthy and time consuming.

An altered steroid hormone metabolism in brook trout (*Salvelinus fontinalis*) treated *in vivo* with 25 ppb cadmium in water for 24 hr. using method I is demonstrated in the autoradiogram (Fig. 1). Both control and treated fish were sacrificed on the 7th day, 5 days after cadmium treatment had ceased, and testicular tissue was incubated with ^{14}C-pregnenolone. It is noted that the yields of steroids from the cadmium treated fish (A) are significantly different from that of the untreated control (B). The results were

confirmed by chemical analysis where there was 24% less conversion of radioactive pregnenolone (spot #7, Fig. 1) by the testicular tissue from the treated fish.

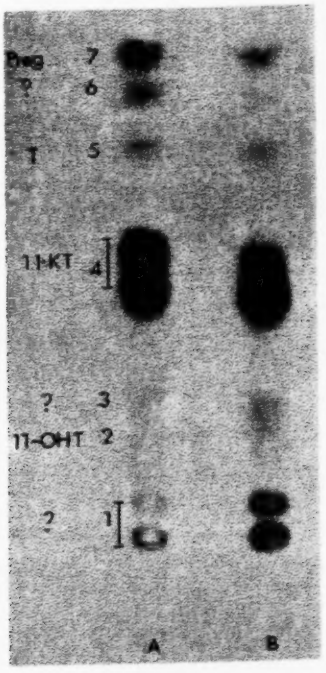

Fig. 1. X-Ray autoradiogram of one-fifth of dichloromethane extracts of Cd-damaged (A) and control (B) testes of brook trout incubated with (4-^{14}C)pregnenolone *in vitro*. 1 to 7 indicate positions of radioactive spots. Areas 1,3, and 6 indicate positions of unknown products; areas 2,4,5, and 7 were isopolar with 11-OHT, 11-KT, testosterone, and pregnenolone, respectively.

Likewise, water may be checked for sublethal effects of pollutants by holding groups of live uncontaminated fish in cages in water or by holding the fish in aquaria containing the water under investigation. Similar groups of fish are held under the same condition in uncontaminated water as controls. As an example, we cite the work of Freeman and Idler, 1975, on the effects of PCB (Aroclor 1254) on brook trout where the toxicity of 0.2 ppm PCB (Aroclor 1254) in water holding brook trout was determined. An autoradiogram was not prepared, however, chemical analysis of the androgens biosynthesized *in vitro* by the male testes showed that PCB's altered steroidogenesis considerably as the biosynthesis of 11β-hydroxytestosterone was nearly double in the PCB treated fish compared to the controls. Eggs from the PCB treated fish fertilized with sperm from the PCB treated fish resulted in only 78% hatching in fresh water compared to the control eggs taken at 100% (Freeman and Idler, 1975).

The effects of contaminants in food may also be determined by carrying out feeding experiments under controlled conditions using two groups of uncontaminated animals, followed by *in vitro* steroidogenesis tests.

The autoradiogram (Fig. 2) demonstrates an altered steroid hormone metabolism by the ovary of a female harp seal (*Pagophilus groenlandicus*) after treatment *in vivo*

with methyl mercury in its diet for 61 days. The autoradiogram was prepared using the neutral fractions of steroid extracts (Freeman *et al.*, 1975). It is noted from the

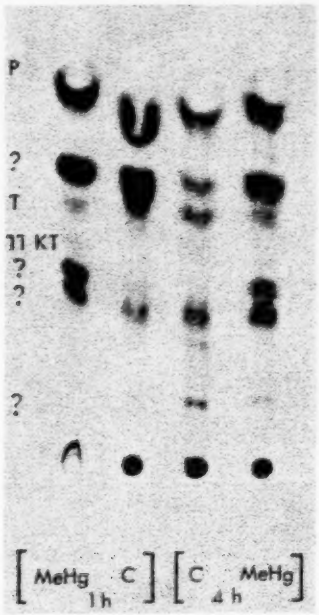

Fig. 2. X-Ray autoradiogram of the chromatogram of 1/10 of the neutral fractions of steroid extracts of ovaries from untreated (C) and methyl mercury treated (MeHg) harp seal. The tissues were each incubated with [^{3}H]-pregnenolone and [^{14}C]-progesterone for 1 h and 4 h *in vitro*. Spots show the positions of radioactive metabolites. Steroid symbols indicate the relative positions of UV fluorescence quenching carrier steroids. ? indicates unknown products.

autoradiogram that the steroid metabolites biosynthesized by the ovary of the treated seal are significantly different from those of the untreated controls at both 1 and 4 hr. It is significant to note that the levels of total mercury in all examined tissues including the ovary were higher in the treated seal than in the untreated seal. There appeared to be no other gross differences in these tissues when examined visually. Histological examination of the ovaries did not reveal any abnormalities under the light microscope. It is interesting that we were able to detect differences in the treated seal due to sublethal levels of methyl mercury in its diets when other methods failed (Freeman *et al.*, 1975).

The effects of a specific contaminant or contaminants may be investigated *in vitro* in the laboratory giving some indication of their *in vivo* effects by incubating the interrenal and/or gonad pools from uncontaminated fish with and without added contaminants and determining any change in steroidogenesis. This is demonstrated in the autoradiogram (Fig. 3) where pooled sections from mid testes from mature untreated brook trout were incubated with increasing amounts of cadmium. An altered steroid

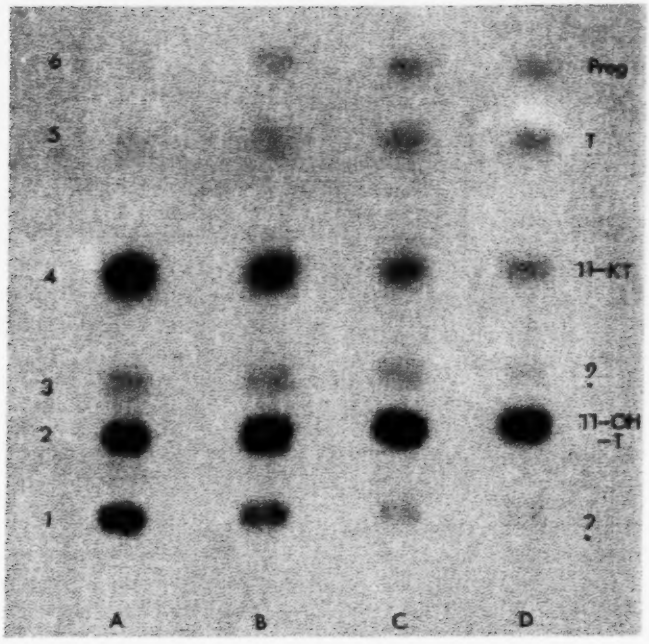

Fig. 3. Autoradiogram of the dichloromethane extracts of normal testes of *S. fontinalis* incubated with [4-^{14}C]pregnenolone in the presence of 0-1000 µg Cd/g tissue *in vitro*. A to D represent one-tenth of 1 g samples incubated with 0, 10, 100, and 1000 µg Cd, respectively. 1 to 6 indicate the positions of radioactive areas. Areas 1 and 3 indicate the positions of two unknown products; areas 2, 4, 5, and 6 were isopolar with radioinert carrier steroids 11-OHT, 11-KT, testosterone, and pregnenolone, respectively.

hormone metabolism occurs and increases as the concentration of the contaminant is increased. It is noted that the concentrations of metabolites isopolar with testosterone, 11ß-hydroxytestosterone and unreacted 4-^{14}C-pregnenolone, increased with the concentration of cadmium when the metabolite isopolar with 11-ketotestosterone decreased.

An altered steroid hormone metabolism (*in vitro*) in the gray seal (*Halichoerus grypus*) is also demonstrated by a number of contaminants added to adrenal incubations (Freeman and Sangalang, 1977), (Fig. 4). It is noted that the metabolite profile is changed considerably by the addition of each contaminant.

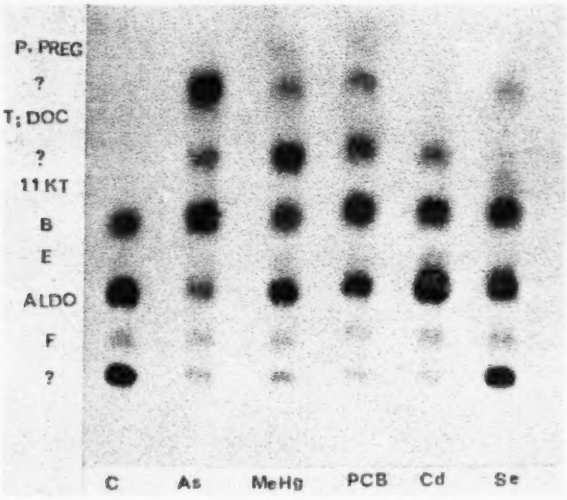

Fig. 4. X-Ray autoradiogram of 1/10 of steroid extracts of adrenal tissues from a gray seal. The tissues were incubated with radioactive steroid precursors [^{14}C]-progesterone and [^{3}H]-pregnenolone with and without contaminants. Spots show the positions of radioactive metabolites. Steroid symbols indicate the positions of UV fluorescence quenching carrier steroids. ? indicates unknown radioactive products. C, control; MeHg, + methyl mercury; As, + arsenic; Cd, + cadmium; Se, + selenium; PCB, + Aroclor 1254.

11.2.5.2 *In Vivo* Tests

The effects of sublethal levels of cadmium on maturation and testosterone and 11-ketotestosterone production *in vivo* in the brook trout are shown in Fig. 5. A DIDA assay method was used to determine testosterone and 11-ketotestosterone levels in blood plasma of male brook trout maintained in fresh water containing 1 ppb cadmium and control fish at various stages of sexual maturation. Plasma testosterone and 11-KT levels correlated to approaching period of functional maturity in control fish, reached a peak of 0.25 and 2.68 ug/100 ml plasma respectively and gradually declined with onset of testicular regression. 11-Ketotestosterone levels of 2.34-3.96 ug/100 ml of plasma in the cadmium treated fish were higher than in the controls and remained elevated during testicular regression. Testosterone levels in the treated fish increased 2 weeks later

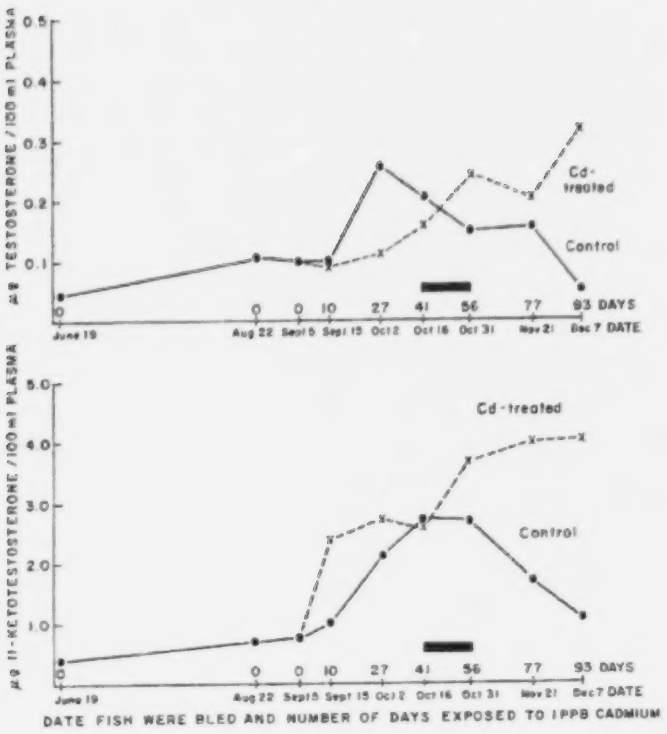
µg TESTOSTERONE / 100 ml PLASMA
0.5
0.4
0.3
0.2
0.1
Cd-treated
Control
0 0 0 10 27 41 56 77 93 DAYS
June 19 Aug 22 Sept 5 Sept 15 Oct 2 Oct 16 Oct 31 Nov 21 Dec 7 DATE
µg 11-KETOTESTOSTERONE / 100 ml PLASMA
5.0
4.0
3.0
2.0
1.0
Cd-treated
Control
0 0 0 10 27 41 56 77 93 DAYS
June 19 Aug 22 Sept 5 Sept 15 Oct 2 Oct 16 Oct 31 Nov 21 Dec 7 DATE
DATE FISH WERE BLED AND NUMBER OF DAYS EXPOSED TO 1 PPB CADMIUM

Fig. 5. Effect of 1 ppb Cd on peripheral plasma levels of testosterone and 11-ketotestosterone in brook trout. Solid bar indicates peak period of functional maturity.

than the control and also remained elevated throughout. The testes of cadmium treated fish regressed at least 2 weeks earlier than the controls. The results suggest an impairment in the clearance and/or utilization of testosterone and 11-ketotestosterone by the cadmium treated fish. The impaired clearance may also be related to the abnormal vasculature and hemorrhagic necrosis observed in the testes of some of the cadmium-treated fish (Sangalang and Freeman, 1974). These results demonstrated how the determination of blood levels of steroid hormones may be used to point out the effects of sublethal levels of contaminants on the reproductive process.

11.3 CONCLUSIONS

In conclusion, it is evident, that sublethal levels of pollutants and contaminants can alter steroidogenesis considerably in fish and animals and therefore, would have an effect on their physiology. Changes in sex steroids and corticosteroid metabolites are particularly useful as indicators for monitoring the effects of pollutants and contaminants on the environment as the enzyme systems involved in steroidogenesis are sensitive to trace levels of these compounds.

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11.5 QUESTION PERIOD

John Davis: As I recall the work we did with Ed Donaldson on the corticosteroids the levels in the plasma were highly transient when we measured stress. Would you like to comment on what this would do to an experimental design using your technique?

H. Freeman: The treated fish would be stressed to a greater degree than the control fish, therefore, one would expect the corticosteroid levels of each group to be different. We have found that trace levels of certain pollutants interfere with the enzyme systems in the biosynthesis of corticosteroids and therefore, the metabolic profile of the corticosteroids shown on the autoradiogram of the treated group would be different from that of the control indicating that there was a problem.

Gilles LaRoche: How long would it take to get an estimate of the effects of a pollutant?

H. Freeman: It would take 2 to 3 days to do a complete *in vitro* steroidogenesis test of a pollutant.

E. Scherer: You presented data illustrating changes in steroid hormone levels brought about by pollutants. Can you extrapolate from these data: what is the significance of these changes with regard to physiology and behaviour of the whole fish? Can you predict, in a reasonably concrete and quantitative way, to what extent survival of the species may be affected by these changes?

H. Freeman: As far as the reproductive physiology is concerned, we can predict in a general way how the species would be affected by determining plasma sex hormone levels. In the present paper we have shown how levels of only 1 ppb of cadmium in water affected the androgen levels in maturing brook trout and have changed the timing of their peaks of functional maturity. It is possible that the males would mature at a different time than the females under these conditions. At higher levels of cadmium, there may be a complete blockage of the reproductive process due to testicular damage.

Jerry Payne: I find the work interesting but there may be a concentration effect. At high concentrations one can get things like tissue necrosis. At lower concentrations like those likely to be found in the environment, it is possible you get an initial induction followed by adaptation.

H. Freeman: In our studies, as far as cadmium is concerned, the testicular damage caused by low levels of cadmium is so extensive that I am sure that adaptation is impossible.

Peter Hodson: Have you related the toxicant concentration in tissues observed after *in vivo* exposures to the effective concentration added to tissues during *in vitro* exposures?

H. Freeman: Yes, we have done some work of this type where we have found similar results, however, much more work of this nature needs to be done.

APPLICATION OF TISSUE CULTURE SYSTEMS TO EVALUATE AQUATIC TOXICANTS

MING FANG LI AND SHEILA CLYBURNE*

Li, M. F. and Sheila Clyburne 1977. APPLICATION OF TISSUE CULTURE SYSTEMS TO EVALUATE AQUATIC TOXICANTS. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5-AR-77-1, Halifax, Canada. pp. 133-140.

Tissue culture cells can be readily propagated under standardized conditions without elaborate facilities and provide a bioassay system, completely avoiding the individual variation inherent in intact animals because of age, sex, reproductive condition, previous environment, and other effects that occur through the life cycle of the individual animal. Various parameters of cultivated cells, such as cellular multiplication, respiration, and morphology are discussed.

Cadmium chloride at a concentration of 0.5 mg/L significantly affected the cellular multiplication. Morphological studies of the experimental cells revealed that $CdCl_2$ severely affected normal mitosis. The abnormality of DNA metabolism could affect the nucleoprotein synthesis and consequently result in a poor stainability of the $CdCl_2$ treated cells by May Grunwald stain.

Li, M. F and Sheila Clyburne 1977. APPLICATION OF TISSUE CULTURE SYSTEMS TO EVALUATE AQUATIC TOXICANTS. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5-AR-77-1, Halifax, Canada. pp. 133-140.

Les cellules de tissus se réproduisent facilement en culture sous un ensemble de conditions hautement standardisées et sans avoir récours à des installations elaborées. Elles se prêtent à un procédé constant d'éssai biologique entierement indépendant des variations individuelles inherentes aux animaux récueillis dans la nature et qui ont trait à l'age, au sexe, à la condition reproductrice, au milieu anterieur et autres facteurs lies au cycle vital des sujets. Divers aspects des cellules cultivées, tels que la multiplication cellulaire, la respiration et la morphologie font l'object de commentaires pertinents.

Le chlorure de cadmium a une concentration de 0.5 mg/L a affecte la multiplication cellulaire de façon significative. L'observation morphologique des cellules soumises à l'éxperience a revele que le $CdCl_2$ affectait serieurement la mitose normale. La situation anormale du metabolisme de l'ADN pourrait affecter la nucleoproteine en synthese dans les cellules traitées au $CdCl_2$.

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12.1 INTRODUCTION

Recent development in vitro cultivation techniques of mammalian and teleostean cells has resulted in advances in both medical and biological sciences. In the past few years the in vitro technique has been frequently suggested for use in toxicity evaluation and environmental studies. Not only can the tissue culture cells be readily propagated under highly standardized conditions without elaborate facilities and provide a constant bioassay system; but also this system avoids the individual variation inherent in intact animals because of age, sex, reproductive condition, previous environment, and other effects that occur through life cycles of the intact organism (Henderson 1960; Lennon 1967; Lennon and Walker 1964; Sprague 1973).

Both mammalian and fish cell cultures have been employed for toxicity evaluation and detection of aquatic pollution. (Gabliks 1965; Gabliks et al 1967; Gabliks and Friedman; Li 1974; Li and Jordan 1969; Li and Traxler 1972; Li et al 1970; Malcolm et al 1973; Metcalfe 1971; Rachlin and Perlmutter 1968, 1969; Richardson 1973; Wilson and Walker 1966).

12.2 MATERIAL AND METHODS

12.2.1 CELL CULTURE

The experimental cells (L-cells, Clone 929) were grown in 50 ml spinner flasks. The spinner modified minimum essential medium (MEM)* was supplemented with 1% fetal calf serum and 0.5% peptone, or with o.5% peptone only. The freshly prepared $CdCl_2$ solution in Hanks' balanced salt solution (HBSS) was added to the spinner medium at a final concentration of 1 mg or 0.5 mg/L. A similar amount of HBSS was added to control cultures.

12.2.2 CELL ENUMERATION AND CYTOLOGICAL STUDIES

The cells were enumerated with an electronic counter (Li and Jordan 1969). The viable cell count and the preparations for light microscopy were performed according to the methods described previously (Li 1974; Li and Traxler 1974; Li et al 1970).

12.3 RESULTS

Figure 1 indicates that the exposure to 0.5 mg/L of $CdCl_2$ in the medium supplemented with 0.5% peptone significantly reduced cellular multiplication to about 50% of that of the control cells in the 4-day experimental period.

In order to evaluate the relative sensitivity of the cells at various growth stages the cells were grown in the medium supplemented with 1% fetal calf serum and 0.5% peptone. Cadmium chloride (1 mg/L) was added at 0, 24, 48 or 72 hours to the various cultures after initiation of the experiment. HBSS was used for control cultures. Figure 2A shows the growth curve of the control cells. After a short lag phase the cells started to multiply logarithmically up to 72 hours. The viability of the cells was most severely affected when the $CdCl_2$ was added at 24 or 48 hours after initiation of the experiment, and an improvement of viability of the cells exposed to $CdCl_2$ at 0 time was observed in the later stages of the experiment (Fig. 2B). Figure 2C shows the overall growth inhibition of the various cell cultures by $CdCl_2$ at the end of the 4-day experimental period. The growth of cultures treated by $CdCl_2$ within 48 hours appeared to have the most serious effect.

The cells from the exposed cultures were harvested and washed once in HBSS, and cultivated in a $CdCl_2$ free medium. Figure 3A shows the recovery of cell viability in the cultures previously exposed to $CdCl_2$ at 0 or 24 hr. The viability of cells treated at 0 hr reached over

* Grand Island Biological Co., 3175 Staley Road, Grand Island, New York.

90% in 4 days. The recovery of cell viability of the cells previously exposed at 24 hr was rather slow. However they did reach 90% viability in the $CdCl_2$ free medium after two successive subcultivations (Fig. 3B).

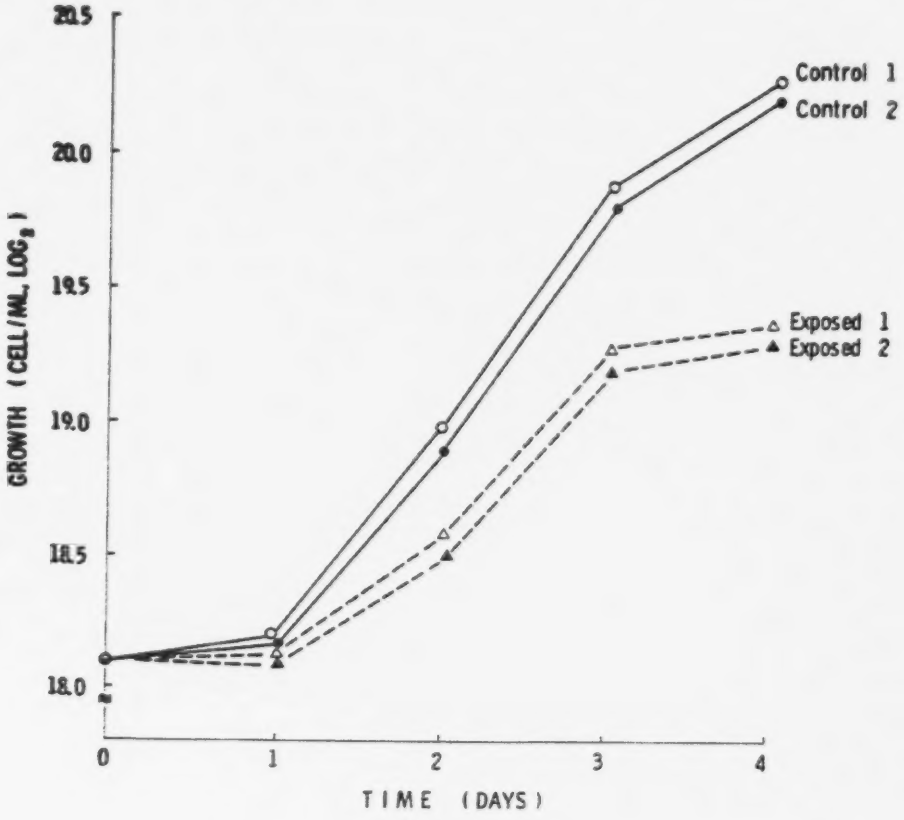

Fig. 1. Effect of cadmium chloride on the growth curve of L-cells grown in spinner MEM medium supplemented with 0.5% peptone at 37 C. Control cells and cells exposed to $CdCl_2$ (0.5mg/L).

In parallel with the growth study of the experimental cells the effect of $CdCl_2$ on cellular morphology was studied also. Figure 4 shows the control cells and the cells exposed at 24 hours after initiation of the experiment. The control cells were uniform in size and well stained (Fig. 4A). For 2 and 3 days $CdCl_2$ exposed cells appeared to lose staining ability and showed cytoplasmic vacuolation and disintegration of the cellular membrane (Fig. 4B and C) in comparison to that of the control cells. Cytotoxic effects of $CdCl_2$ were further demonstrated by Feulgen staining of the experimental cells (Fig. 5). The control cells show well defined nuclei and even distribution of chromatin (Fig. 5A). The nuclei of the experimental cells show pyknosis, aggregation of chromatin and the tendency to divide or fragment after two days exposure to $CdCl_2$ (Fig. 5B). The cells apparently underwent amitosis and became multinucleated (Fig. 5C).

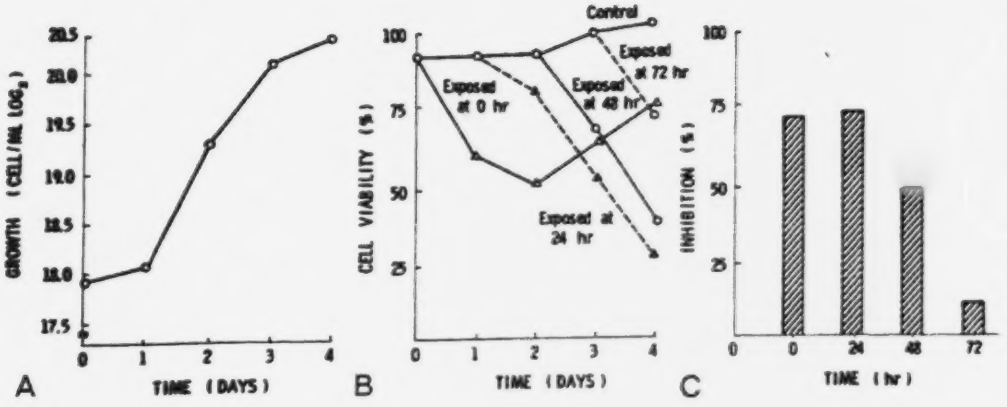

Fig. 2. Effect of cadmium chloride on relative sensitivity of L-cells at various growth phases in the MEM medium supplemented with 1% fetal calf serum and 0.5% peptone at 37 C. A, Growth curve of the control cells; B, Cell viability of the experimental cells when $CdCl_2$ (1 mg/L) was added to the various cultures at 0, 24, 48 or 72 hr after initiation of the experiment; C, Percent inhibition of the $CdCl_2$ treated cells in comparison with control cells at the end of the 4-day experimental period.

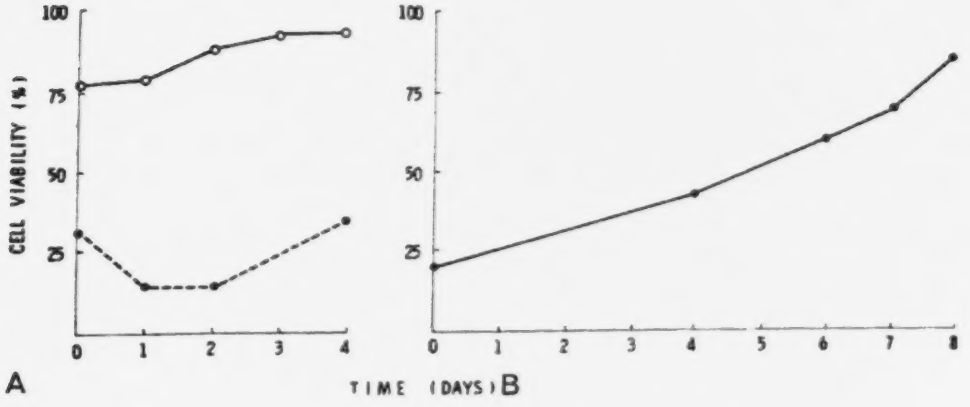

Fig. 3. Recovery of percent cell viability of the previously $CdCl_2$ exposed cultures in the medium containing no $CdCl_2$. A, ●---●, the culture previously treated by $CdCl_2$ at 0 hr; o—o, the culture previously treated by $CdCl_2$ at 24 hr after initiation of the experiment; B, further recovery of the culture previously treated at 24 hr after two more subcultivations.

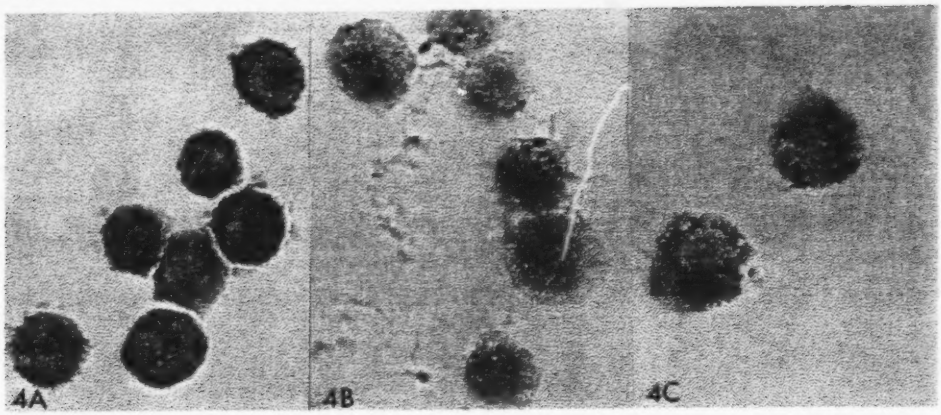

Fig. 4. Control cells and cells treated with $CdCl_2$ (1 mg/L) 24 hours after start of experiment. A. Control cells, well stained and uniform in size at start of experiment. B. Treated cells, after exposure to $CdCl_2$ for 2 days. Cells fail to stain well. C. Treated cells, after exposure to $CdCl_2$ for 3 days. Cytoplasm is vacuolated and cell membrane is poorly defined.

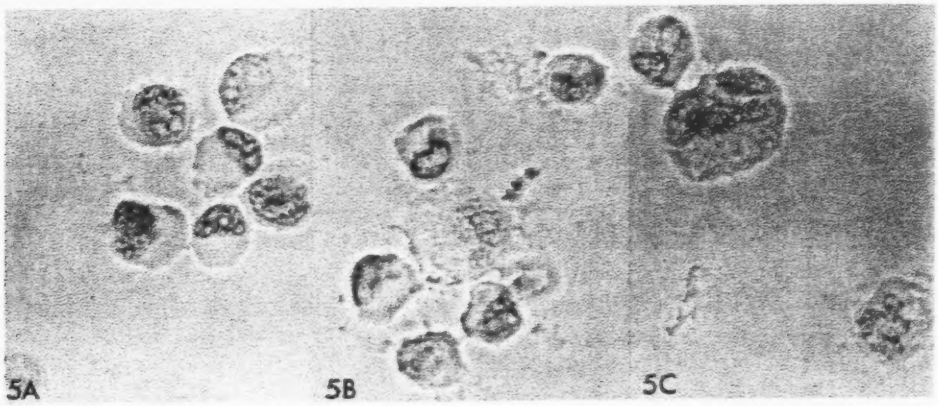

Fig. 5. Control cells and cells treated with $CdCl_2$ (1 mg/L) 24 hr after start of experiment. A. Control cells with even distribution of chromatin; B. Treated cells after exposure to $CdCl_2$ for 2 days. Note the hyperchromatic nuclear membrane, clumped chromatin and fragmentation or division of nucleus; C. Treated cells, after exposure to $CdCl_2$ for 3 days. Multinucleated cells have developed. Feulgen stain x 410.

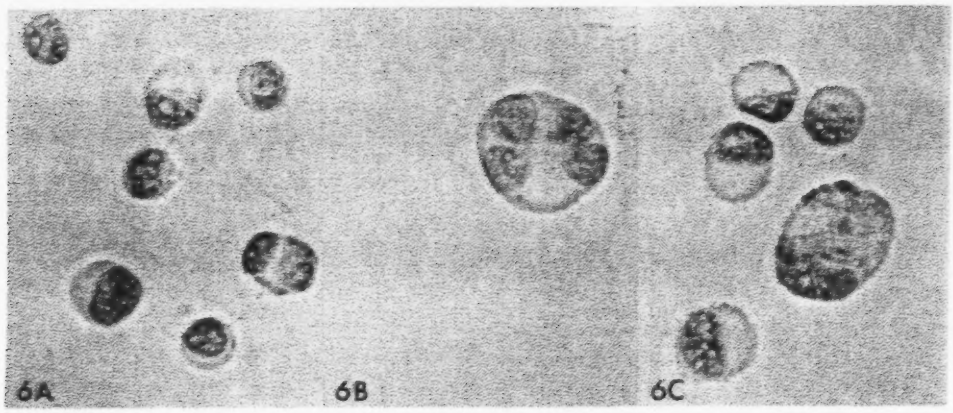

Fig. 6. Cells, subcultured in $CdCl_2$ free medium following exposure to $CdCl_2$. A. Cells after 4 days in $CdCl_2$ free medium following 4 days exposure to $CdCl_2$ from start of experiment (0 hr). Most cells showed normal morphology. B. Cells after 4 days in $CdCl_2$ free medium following 3 days exposure to $CdCl_2$ starting 24 hr after initiation of experiment. Abnormal cells were still present in culture. Note the multinucleated giant cell. C. Same cells as in B but 2 further subcultivations of 4 days each. Many cells now showed normal morphology. Feulgen stain x 410.

Figure 6 shows cellular morphology of the previously exposed cells after cultivation in the medium containing no $CdCl_2$ for a period of time. After 4 days the majority of the cells previously exposed at 0 hr had normal morphology (Fig. 6A), and the giant and multinucleated cells were still frequently observed in the culture previously exposed at 24 hr (Fig. 6B). After 2 more successive subcultivations the majority of the cells showed normal morphology (Fig. 6C).

12.4 DISCUSSION

Serious limitations exist in bioassay techniques for monitoring overallquality of water with intact animals (Lennon and Walker 1964); however cell culture systems allow many variables to be controlled, and provide a wide range of biochemical, physiological, and cytological studies of the biological effects of pollutants (Li 1974; Li and Jordan 1969; Li and Traxler 1972, 1974; Li et al 1970; Rachlin and Perlmutter 1968). Fish cells were found to be

more susceptible than intact animals (Rachlin and Perlmutter 1968, 1969), and suspension cell cultures responded with greater sensitivity to $HgCl_2$ than did fish cell cultures (Li 1974; Li and Jordan 1969; Li and Traxler 1972). The present study demonstrates that suspension cell cultures are highly sensitive to cadmium (Bahner and Nimmo 1975; Malcolm et al 1973). The slight variation observed in the duplicate cultures (Fig. 1) suggest the reliability of the bioassay system. This may further reflect the advantage of using cultivated cells to avoid variability of individual organisms and environmental conditions of intact animal bioassay systems. However, many factors, such as growth phase of stock culture, culture medium, and initial cell density could affect the sensitivity of the culture system to the pollutants (Li and Jordan 1969; Li and Traxler 1972), so that a control culture must always be used in the present bioassay system.

For evaluation of the relative sensitivity of the cells (and their morphological changes) at various growth phases, 1% fetal calf serum was incorporated into the experimental medium in order to avoid totally lethal effects of $CdCl_2$ in the cultivated cells (Li and Traxler 1972). Both cell viability and growth inhibition studies suggest the cells during the logarithmic stage were most sensitive to the toxicity of $CdCl_2$ (Fig. 2).

The information obtained from morphological studies indicates that nuclear or DNA metabolism of the exposed cells was affected by $CdCl_2$ (Fig. 4, 5, and 6) resulting in interruption of normal mitosis and formation of multinucleated cells. The abnormality of DNA metabolism could also affect nucleoprotein synthesis and result in a reduced staining ability of the $CdCl_2$ exposed cells.

The experimentally exposed cells appear to be able to recover from injury (Fig. 3 and 6). However, severely injured cells at the active growth phase appear to take a longer time to recover. Further investigation of the exact cellular mechanism of injury repair is needed.

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12.6 QUESTION PERIOD

Peter Hodson: We would like to regulate materials in water and you are adding the materials to tissue culture. Have you ever related the concentrations that are effective in tissue culture to those effective in fresh water so that we could use your results to relate to water quality?

Ming F. Li: Not yet since the tissue culture bioassay method is rather new to environmental studies. More research and more work on calibration with the in vitro studies on toxicants are obviously needed. According to the limited information the cell cultures are highly sensitive to pollutants, and they provide a wide range of information regarding the specific activity of pollutants at the cellular level. Any ill effects of polluted water regardless of source should be reflected in the cell system reactions.

Ed Pessah: As an extension of Peter Hodson's comment, isn't an intermediate step required that relates the concentration of toxicants causing tissue damage to the concentration in the organism required to cause the damage? One could then determine the concentration in the water needed to elicit the effect in the organism and that could be used as a basis for developing safe concentrations.

Ming F. Li: Yes, I believe there is an intermediate step which could involve the accumulation or detoxification of the toxicants by the intact animal and should be determined separately for the different species and contaminants. The intermediate step would be minimized in the cell culture system, consequently the cell system should give more constant assessment of the environmental quality.

Peter Wells: Have you ever considered using protozoa in these types of tests?

Ming F. Li: Only for scientific interest because from the practical point of view the need is to develop a bioassay system and I believe that the intact animal and/or cell culture should be used. As indicated in my report, the cell culture system has great potential in the future to assess our environmental conditions.

COMPARISON OF RAPID BIOASSAY PROCEDURES FOR MEASURING TOXIC EFFECTS OF BLEACHED KRAFT MILL EFFLUENT TO FISH

DONALD J. MCLEAY AND TERENCE E. HOWARD*

McLeay, D.J., and T.E. Howard. 1977. COMPARISON OF RAPID BIOASSAY PROCEDURES FOR MEASURING TOXIC EFFECTS OF BLEACHED KRAFT MILL EFFLUENT TO FISH. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5-AR-77-1, Halifax, Canada. pp. 141-155.

Comparisons were made between seven sublethal bioassay procedures that have been developed in part as alternatives to the conventional 96-h LC50 bioassay for measuring the acute toxicity of bleached kraft pulpmill effluent (BKME). The fish tested were juvenile coho salmon [*Oncorhynchus kisutch*]; weight 0.82±0.15g, length 4.25±0.21cm. The study was carried out within seven days using a single 3400-ℓ sample of BKME collected from a British Columbia coastal mill during normal operations. The initial 96-h LC50 value of 15.0% v/v for the neutralized filtered effluent remained essentially unchanged at 15.8% v/v by the end of the study. Concentrations of BKME used in the sublethal bioassays were expressed as proportions of the initial 96-h LC50 value.

The responses measured were: elevated blood sugar levels, decreased red blood cell counts, decreased white blood cell-thrombocyte counts, reduced upper lethal temperatures, elevated residual oxygen levels (sealed jar bioassays using both high and low fish loadings), and decreased critical swimming speeds. Threshold effect (EC50) values for these bioassays were respectively 0.04, 0.6, 0.1, 0.3, 0.9, 0.4 and 0.5 of the LC50, after test durations of 4, 24, 24, 19, 4.5, 12 and 27 h. The various procedures were also compared in terms of effluent volume, labor and special equipment requirements.

McLeay, D.J., and T.E. Howard. 1977. COMPARISON OF RAPID BIOASSAY PROCEDURES FOR MEASURING TOXIC EFFECTS OF BLEACHED KRAFT MILL EFFLUENT TO FISH. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5-AR-77-1, Halifax, Canada. pp. 141-155.

On a comparé sept méthodes d'éssais biologiques, à des taux de toxicité non mortels, élaborés en partie comme substitut à l'epreuve du LC50** en 96 heures pour mesurer la toxicité aiguë des effluents des usines de papier kraft blanchi (EUPKB). On a pris pour sujets d'experience de jeunes saumons

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**Lethal concentration 50 percent (niveau de concentration provoquant 50 p. 100 de décés).

coho, *Oncorhynchus kisutch*. Leur poids était de 0,82±0,15g et leur taille, de 4,25±0,21cm. L'étude s'est déroulée en une période de sept jours, consommant un seul échantillon de 3400 litres d'EUPKB prélevé à une usine côtière de la Colombie-Britanique dans le cours normal de son exploitation. La valeur de 15% par volume d'éffluent filtré et neutralisé, de l'épreuve initiale du LC50 en 96h est demeure pratiquement inchangee, atteignant 15,8% a'la fin des essais. Les concentrations d'EUPKB servant aux essais biologiques non mortels ont été experimées comme proportions de la valeur initiale de l'épreuve du LC50 en 96 hr.

Pour chaque méthode d'éssai respectif on a pu observer: Une augmentation du taux de glycémie, une diminution du nombre de globules rouges, une diminution du nombre de globules blancs (thrombocytes), une reduction de seuil critique des temperatures maximum permettant la survie des poissons, des niveaux élevé d'oxygène résiduel (éssais en vase clos utilisant de fortes et de faibles densités de poisson par volume de solution d'éssai), et une diminution de la vitesse critique de natation. Les valeurs du seuil de manifestation (EC50)* se sont établies respectivement à 0,04., 0,6., 0,1., 0,3., 0,9., 0,4., et 0,5 de celles de l'epreuve du LC50 pour des durées d'essai de 4., 24., 24., 19., 4,5., 12., et 27 heures. Le volume d'éffluent nécessaire, la somme de travaile et l'équipement spécialisé requis ont également servi de base de comparaison pour les divers procédés.

13.1 INTRODUCTION

A number of techniques are now available for quantitatively assessing the acute sublethal toxic effects of pulpmill effluents to fish. Included are tests which determine the threshold effect concentrations which impair swimming performance; decrease the upper lethal temperature; alter the numbers of circulating blood cell types; evoke a hyperglycemic stress response and decrease the tolerance of fish to oxygen reduction in sealed jar bioassays (Howard 1973, 1975; McLeay 1975, 1976, 1977). Such tests have considerable advantages over the conventional 96-h LC50 bioassay concerning sensitivity, test duration and effluent volume requirements; and are particularly valuable for rapidly assessing harmful levels of effluents which as a result of mill treatment are not acutely lethal.

The performance of the above sublethal tests has not been compared. Since the test responses are susceptible to variations in the condition of the fish stock used and the nature of the toxic constituents, a meaningful assessment of the virtues of new bioassay procedures requires that the tests be conducted simultaneously, using the same fish stock and toxicant. In the present study, the performance of a blood sugar bioassay, red blood cell and white blood cell-thrombocyte count bioassays, temperature tolerance test,

* Effective concentration 50 percent (niveau de concentration ou commence a se manifester l'une des modifications de comportement).

sealed jar bioassays and a swimming performance test were compared with a 96-h LC50 batch-replacement bioassay (APHA 1975). All tests were carried out concurrently using a single population of juvenile coho salmon and a single sample of neutralized, filtered, bleached kraft mill effluent.

13.2 MATERIALS AND METHODS

13.2.1 Fish and Water Supply

Coho salmon (*Oncorhynchus kisutch*), obtained as fry from the Capilano Hatchery (Environment Canada) in British Columbia during April 1975, were maintained at B.C. Research for 1 month prior to testing. During this time the fish were held under natural illumination at low population density in a 3.1-m diameter fibreglass tank receiving dechlorinated Vancouver City tap water. Water quality characteristics (mean ±SD values) included pH 6.6 ± 0.05, temperature 11 ± 0.6 C, conductance 13.8 ± 1.8 umho/cm, alkalinity 4.4 ± 0.6 mg $CaCO_3/\ell$ and EDTA hardness 6.2 ± 0.3 mg $CaCO_3/\ell$. This water was used as dilution water for all bioassays.

Fish were fed five times daily with Oregon Moist Pellets (Moore-Clark Ltd., LaConner, Washington). At the time of the studies, the fish measured 4.25 ± 21 cm fork length and weighed 0.82 ± 0.15 g.

13.2.2 Effluent and LC50 Bioassays

A 3400-ℓ sample of whole bleached kraft mill effluent (BKME) was collected from a coastal British Columbia mill during a period of normal operation. The sample was transported in full, sealed polyethylene containers to our laboratory where it was mixed thoroughly in a large fibreglass tank, filtered to remove pulp fiber and then stored at 4 C in full, sealed polyethylene barrels. Aliquots for LC50 and sublethal bioassays were heated to 11 ± 0.5 C, adjusted to pH 6.6 ± 0.05 with NaOH or H_2SO_4, and oxygenated to saturation level. All tests were completed within 7 days of sample collection.

The toxicity of this effluent sample to the fish was measured by 96-h LC50 bioassay (APHA 1975), initially and at termination of the sublethal tests. During these bioassays, fish loading was 0.5 g fish/ℓ/day, and the test solutions were replaced every 24 h. These bioassays were carried out in rectangular containers (45 X 35 X 40 cm) with minimal aeration to maintain the dissolved oxygen content of test solutions at 9 mg O_2/ℓ.

All tests except the sealed jar bioassays were conducted at 11 ± 0.5 C within controlled temperature rooms. Unless otherwise stated fish were not fed for any bioassay during the 24 h preceding effluent exposure nor during the period of exposure.

13.2.3 Blood Sugar Test

Basic test procedures were those described by McLeay (1977). Groups of twelve fish were transferred from the stock tank to six polyethylene tanks, 45 X 35 X 40 cm,

fitted with opaque lids and each containing 22 liters of fresh water. The fish were not fed after transfer nor for 16 h prior to this time. Following a 24-h acclimation period, effluent was added carefully in a manner which caused minimal disturbance to the fish and concentrations equivalent to 0.05, 0.1, 0.2 and 0.3 of the initial 96-h LC50 value. At this time two control tanks received additional volumes of water equivalent to the lowest and highest effluent concentrations. Effluent or water was added at 20-min intervals to the individual tanks to allow time for the collection of blood samples. After a 4-h exposure to effluent, all fish in each tank were sampled sequentially within 12 min lapsetime and their blood collected from the severed caudal peduncle into individual heparinized micro-hematocrit tubes. After centrifugation, the blood plasma was stored at -20 C until analysed for glucose content according to Dubowski (1962).

13.2.4 Blood Cell Count Tests

Twelve fish were placed in each of eight plexiglass compartments, 200 X 12 X 20 cm, which received a continuous supply (0.25 ℓ/min/tank) of fresh water at 11 ± 0.5 C (McLeay 1975). A current sufficient to cause fish orientation was maintained in each compartment by recirculating the solution at 10 ℓ/min. During a 2-week acclimation period, the fish were fed three times daily while excess food and feces were removed by siphoning. Subsequently, the fish were exposed to BKME for 24 h at concentrations equivalent to 0 (control), 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 1.0 of the initial 96-h LC50 value. Effluent or water (control) was added continuously to the stream compartments from 110-ℓ polyethylene cylinders via peristaltic pumps at a rate which provided 2 ℓ fresh solution/g fish/24 h (Sprague 1969).

Following 24-h exposure to effluent, all fish were sacrificed for red blood cell (RBC) and white blood cell-thrombocyte (WBC-T) counts according to techniques described previously by McLeay (1973).

13.2.5 Temperature Tolerance Test

Basic procedures were those described by Howard (1973). Twenty fish were added to each of 13 rectangular polyethylene tanks containing effluent at concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.4 and 1.6 of the initial 96-h LC50 value. Twenty fish were placed in each of two additional tanks containing water only (controls). The tanks were positioned randomly in a controlled temperature room free from disturbances. Fish loading in all tanks was 0.5 g/ℓ. The temperature of each test solution was initially 11 ± 0.5 C and was increased progressively at 1 C/h. The temperature at time of death of each fish was recorded. Exposures were continued until the last fish died (within 19 h).

13.2.6 Sealed Jar Bioassays

Test procedures detailed by McLeay (1976) were followed. The tests were conducted in 0.92-ℓ glass jars at ambient laboratory temperature (21.7 ± 0.3 C). Required volumes of effluent and dilution water were heated to 22 C and their dissolved oxygen content adjusted to the saturation level for this temperature. The jars were filled with test solutions; fish were added and the jars were filled completely and sealed. The residual oxygen levels at time of death of the last (or only) fish in each jar were measured using a portable oxygen analyzer probe and attached stirrer (Delta Scientific Ltd.) inserted into the jar.

The sensitivity of this test was examined with both high (4 fish/jar; 4 g/ℓ) and low (1 fish/jar; 1 g/ℓ) fish loadings, using 10 replicate jars for each concentration. Concentratons tested were 0, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 of the initial 96-h LC50 value for the high loading; and 0, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 1.0 LC50 for the low loading.

13.2.7 Swimming Performance Test

Critical swimming speeds (Brett 1964, 1967) of coho salmon in fresh water or in BKME were determined at 11.0 ± 0.5 C, using a three-tunnel stamina testing apparatus (Howard 1973). Test procedures were according to Howard (1975). Critical swimming speed (Vcrit) was measured in lengths per second (L/s). Initial velocity was 0.8 L/s and the speed was increased at 60-min intervals in steps of approximately 0.8 L/s until the fatigue velocity. The Vcrit for each fish was calculated according to Brett (1964). The concentrations of effluent to which fish were exposed for 18 h prior to exercise and during the swimming test were 0 (control), 0.3, 0.4, 0.5 and 0.6 of the initial 96-h LC50 value. Thirty fish (10/tunnel) were tested in each effluent concentration. Fish loading during the 18 h pre-exercise period was 0.5 g/ℓ.

13.2.8 Statistical Analyses

The 96-h LC50 values, together with 95% confidence limits, were calculated according to APHA (1975) and Litchfield and Wilcoxon (1949). The median effective concentration (EC50), expressed as a proportion of the initial LC50 value, was estimated for each sublethal bioassay. The proportion of fish showing a net significant response at each concentration was determined and the EC50 value and its confidence limits calculated by log-probit analysis according to Sprague (1968) and APHA (1975).

In the blood sugar and temperature tolerance tests, values for the two control groups were compared by a 2-tailed Student's t-test. Since control groups in either test did not differ ($P>0.05$), values were pooled for comparison with the effluent treatments.

13.3 RESULTS AND DISCUSSION

The chemical characteristics determined for the effluent sample were pH 3.0, conductance 1450 µmho/cm, BOD_5 235 mg/ℓ, sodium 292 mg/ℓ and color 2200 APHA units. The initial and final 96-h LC50 values for the neutralized effluent were 15.0 (14.2, 15.9) and 15.8 (14.8, 16.5)% v/v respectively (95% confidence limits in parentheses). Thus the toxicity of the effluent was essentially unchanged throughout the duration of the sublethal bioassays.

Mean (±95% confidence interval) values obtained for the various BKME concentrations in each sublethal bioassay are illustrated in Figures 1-7. Threshold effect (EC50) concentrations, test durations, effluent volume requirements (expressed as portions of that required for the LC50 bioassay), labor and special equipment requirements for each test are summarized in Table 1. The EC50 values ranged from 0.04 LC50 for the blood sugar test to 0.9 LC50 in the sealed jar bioassay with high fish loading. The periods for which fish were exposed to effluent varied from 4 or 4.5 h in the blood sugar test and sealed jar bioassay with high loading to 27 h in the swimming performance test. Effluent volume requirements ranged from 2-3% of that used in the 96-h LC50 test for the blood sugar and sealed jar (high loading) bioassays to 80% in the swimming stamina test. Total labor requirements for each sublethal bioassay varied from 10 to 55 man-h compared with approximately 14 man-h for the LC50 bioassay (Table 1).

The blood sugar test (Figure 1) had a number of advantages over the other procedures examined, including the best sensitivity (EC50 0.04 of the LC50 value), the shortest exposure period (4 h) and the least effluent volume requirements. Total labor requirements including chemical analyses for plasma glucose were similar to those for the LC50 bioassay. Special equipment required for this test included a spectrophotometer with a microcell adapter.

The technique employed for analysis of blood sugar (Dubowski 1962) is simple and inexpensive; automated glucose micro-analysers are available which would decrease the labor requirements for analysis considerably. The major disadvantage of this test is that the extent to which blood sugar levels are elevated due to pulpmill effluent exposure appears to be affected by the glycogen energy stores of the fish (McLeay 1977), which may vary seasonally and which are altered markedly by culturing techniques (McLeay, unpublished results). Another disadvantage is that a hyperglycemic response caused by increased secretions of glucocorticoid and catecholamine stress hormones (Falkmer 1961; Young and Chavin 1965) is evoked by any stressor (Narasimhan and Sundararaj 1971; Wedemeyer 1972). Therefore the test is susceptible to extraneous disturbances and must be performed under strictly controlled conditions.

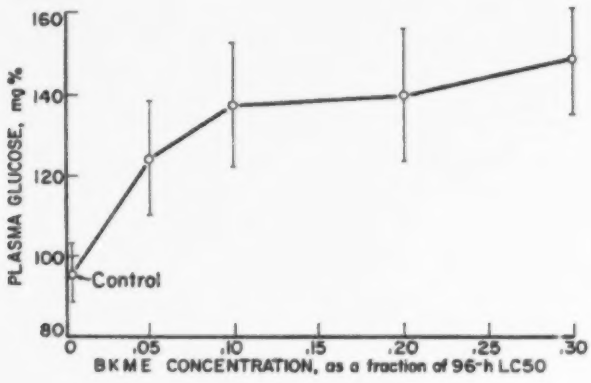

Fig. 1. Plasma glucose levels in coho salmon exposed to BKME for 4 h after a 24-h acclimation.

Red blood cell counts were decreased by a 24-h exposure to 0.6 and 1.0 LC50 strengths of pulpmill effluent, and not affected by lower concentrations (Figure 2). A similar decrease in RBC counts due to 24-h exposure to high sublethal BKME concentrations only was found previously (McLeay 1975). Since the response of RBC counts to pulpmill effluent is relatively insensitive (EC50, 0.6 LC50 in the present study), and since this test has considerable effluent volume and labor requirements, RBC counts should not be considered further as a useful method for routinely assessing the acute toxicity of pulpmill effluents.

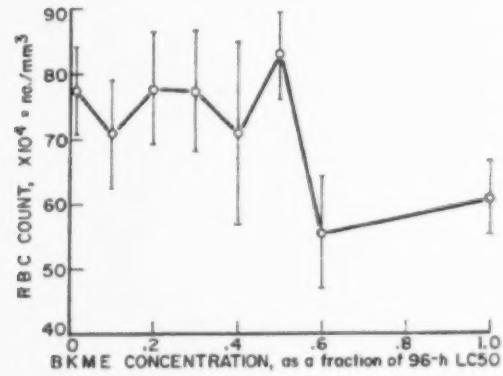

Fig. 2. Red blood cell counts in coho salmon exposed to BKME for 24 h.

Mean WBC-T counts were decreased by 24-h exposure to all BKME concentrations tested (Figure 3). The estimated EC50 value was 0.1 LC50 (Table 1). In a previous study

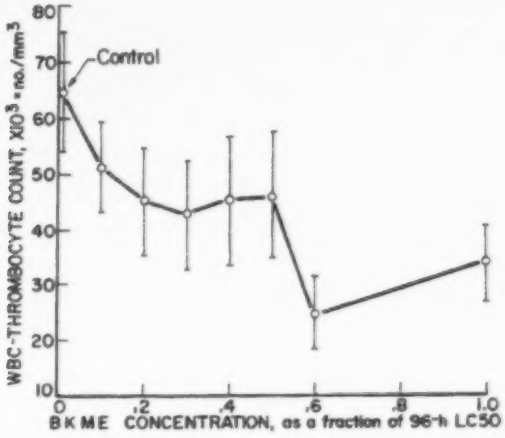

Fig. 3. White blood cell-thrombocyte counts in coho salmon exposed to BKME for 24 h.

(McLeay 1975), WBC-T counts were decreased significantly in coho exposed for 24 h to BKME concentrations of 0.2 LC50 and higher, as well as by other situations considered as stressful. Thus the WBC-T count test appears to be a reliable, sensitive and reasonably rapid method for determining stressful levels of pulpmill effluents to coho salmon, with a small effluent volume requirement (0.1 of the volume needed for the 96-h LC50 bioassay). Since this response is stress-induced, rigid control of external influences during the test period is required. A disadvantage of the WBC-T count test according to present practises is the labor involved (28 man-h) during acclimation of fish to stream compartments for two weeks prior to effluent exposure. Although this procedure ensures that the fish are fully recovered from the stress due to transfer, previous results (McLeay 1975) suggest that this period could be reduced to one week or less. Further studies are required to increase the efficiency of this test.

In the temperature tolerance test, mean lethal temperatures decreased progressively with increasing BKME concentrations (Figure 4). The EC50 value of 0.3 LC50 (Table 1) is similar to that for other BKME samples tested under identical conditions (McLeay, unpublished data). The test is completed in less than 24 h, and requires only 10% of the effluent volume needed for a 96-h LC50 batch-replacement bioassay (Table 1). Labor

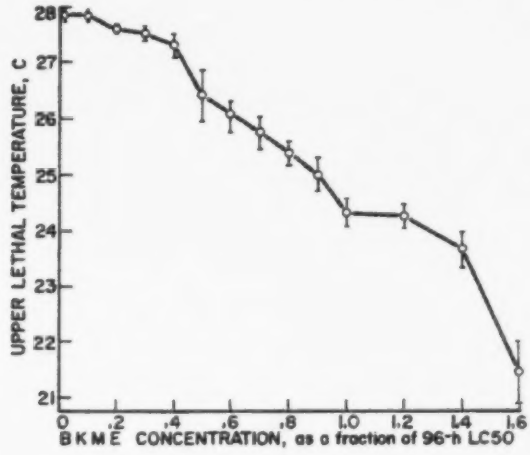

Fig. 4. Upper lethal temperatures for coho salmon exposed to BKME.

requirements are similar to those for the LC50 bioassay. This test appears to be less sensitive than the blood sugar or WBC-T count stress responses, but more sensitive than the swimming performance test, RBC count test or sealed jar bioassays.

The sealed jar bioassays with fish loadings of 4 g/ℓ (Figure 5) or 1 g/ℓ (Figure 6) showed progressive increases in mean residual oxygen levels with increasing BKME concentrations. When conducted at a low loading (1 g/ℓ), the sealed jar bioassay

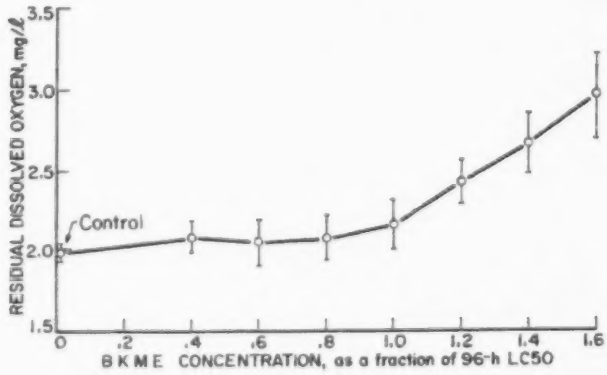

Fig. 5. Residual dissolved oxygen levels at death of coho salmon exposed to BKME at 20 C; fish loading 4 g/ℓ.

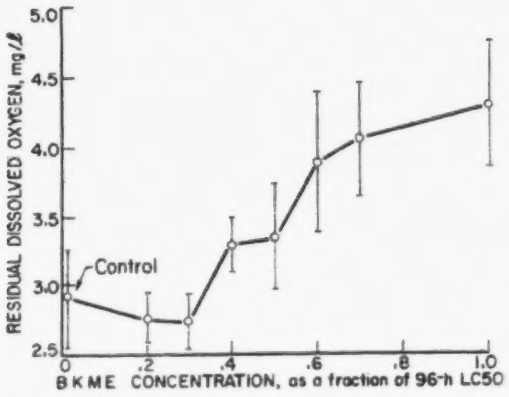

Fig. 6. Residual dissolved oxygen levels at death of coho salmon exposed to BKME at 20 C; fish loading 1 g/ℓ.

is completed within 12 h, requiring only 5% of the effluent volume and approximately the same man-h of labor needed to complete the 96-h LC50 bioassay. The threshold response concentration to BKME (0.4 LC50) at this loading was similar to that found previously for coho salmon (McLeay 1976). At a high loading (4 g/ℓ) the sealed jar bioassay is somewhat less sensitive (threshold response concentrations for coho salmon 0.5-0.9 LC50 in this and other [McLeay 1976] studies); however the test is completed within 5 h using even less effluent (3% of LC50 requirements) and man-h (Table 1). The sealed jar bioassay is applied at ambient laboratory temperature while the sole special equipment required is a dissolved oxygen meter.

In the swimming performance test, mean critical swimming speeds decreased with increasing BKME concentrations (Figure 7). The EC50 value of 0.5 LC50 (Table 1) is similar or slightly higher than threshold effect concentrations for other BKME samples where the LC50 values were derived from replacement (2 ℓ/g/day) bioassays (unpublished data). Thus this test is relatively insensitive compared with the blood sugar, WBC-T count or temperature tolerance tests. Other shortcomings include its sophisticated equipment and high labor requirements, with little reduction in effluent volume requirement from that of the LC50 bioassay. Obviously, this test in its present form was not designed for routine monitoring of toxic-effect concentrations. Nonetheless measurement of the threshold level at which treated or untreated pulpmill effluents affect swimming performance should be continued in programs designed to assess safe limits of discharge to the environment, since impairment of swimming performance is an ecologically significant response to pollutants. A modified multi-channel swimming apparatus could reduce effluent volume and labor requirements for this test considerably.

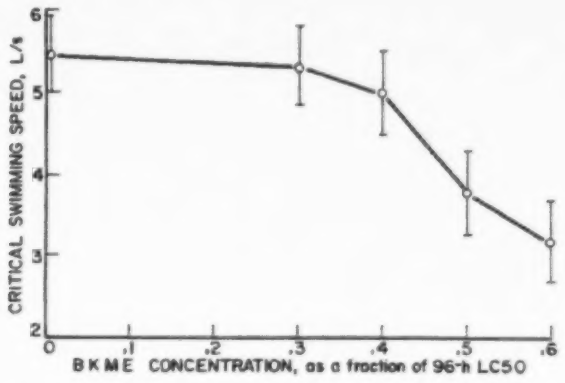

Fig. 7. Critical swimming speeds for coho salmon exercised in BKME after an 18-h pre-exposure.

Table 1. Intercomparison of bioassay procedures for measuring acute toxic effects of pulpmill effluents[a] to coho salmon.

Test	EC50[b] (portion of 96-h LC50)	Exposure period	Effluent volume[c]	Labor (man-h)[d]	Special equipment
blood sugar	0.04 (0.004,0.2)	4	0.02	14	spectrophotometer
RBC count	0.6 (0.5,1.1)	24	0.6	48[e]	compound microscope
WBC-T count	0.1 (0.02,0.2)	24	0.1	48[e]	compound microscope
upper lethal temperature	0.3 (0.2,0.3)	19	0.1	14	controlled variable temperature room
residual oxygen (high fish loading)	0.9 (0.8,0.9)	4.5	0.03	10	dissolved oxygen meter
residual oxygen (low fish loading)	0.4 (0.3,0.5)	12	0.05	14	dissolved oxygen meter
critical swimming speed	0.5 (0.4,0.5)	27[f]	0.8	55 (11 per concentration)	swimming apparatus
96-h LC50 (2 ℓ/g/day)	1.0	96	1.0	14	-

[a] based on simultaneous testing with one large grab sample of filtered, pH-adjusted bleached kraft mill effluent.

[b] median effective concentration (concentration causing a response in 50% of the test fish).

[c] calculated as portion of volume of effluent required for a 96-h, batch-replacement LC50 bioassay with 2 liters of test solutions/g fish/day.

[d] includes set-up and clean-up time, and time required for carrying out the test analysis and data tabulation.

[e] includes 28 h for fish maintenance during 2-week acclimation.

[f] includes 18 h pre-exposure to effluent prior to swimming test.

The sublethal tests described all have increased sensitivities and decreased test durations than the 96-h replacement LC50 bioassay. Although continuous flow-through 96-h LC50 bioassays provide a similar or identical measure of the acute toxicity of pulp-mill effluents as batch-replacement LC50's with the same exchange rate (Walden et al. 1975), flow-through bioassays have a greater effluent volume and special equipment requirement. Therefore assessment of the present sublethal bioassay procedures in relation to a continuous-flow LC50 bioassay would further enhance the potential of sublethal bioassay tests of the type described here.

There is an increasing awareness that determining and establishing safe limits for effluent discharge will require measurements of various meaningful sublethal toxic responses of fish and other aquatic organisms. Such responses must include sensitive stress indicators, respiration effects and other responses of obvious ecological significance such as impaired swimming performance (fish) and alterations of the zones of tolerance of the organisms to temperature and dissolved oxygen extremes. Sublethal tests such as those compared in this study meet most of the required criteria and provide a variety of methods to rapidly assess the effects of pulpmill effluents in the environment. In addition, new techniques need to be developed to determine the broad spectrum of relevant biological responses of fish and other aquatic organisms to these pollutants.

13.4 ACKNOWLEDGEMENTS

This study was sponsored by British Columbia Forest Products Ltd., Canadian Forest Products Ltd., Crown Zellerbach Canada Ltd., MacMillan Bloedel Ltd., Northwood Pulp and Timber Ltd., Prince George Pulp and Paper Ltd., Tahsis Company Ltd., and Weyerhaeuser Canada Ltd. The technical assistance of Messrs. M.R. Gordon and J.R. Munro is gratefully acknowledged.

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13.6 QUESTION PERIOD

John Sprague: I would like to compliment you on your paper. Have you abandoned the coughing response?

Don McLeay: No; however it has a number of major drawbacks as a routine monitoring bioassay. It requires sophisticated equipment and the fish acclimate to the effluent. Also, the performance of individual fish varies quite considerably. We excluded it from this series of comparisons due to time and man-power restrictions.

Garth Fletcher: Do you know the reason for the drop in red blood cell counts? Is it the result of cell destruction or an increase in blood volume? If there is an increase in blood volume the fish may be having an osmotic problem and other indicators of osmotic imbalance may precede the RBC changes. Destruction of RBC's may also indicate osmotic imbalance.

Don McLeay: I believe that this response is due to destruction or increased fragility of the erythrocytes. In this and other studies where fish were exposed to high sublethal concentrations of pulpmill effluents we often observed contamination of plasma or serum with hemoglobin due to cell lysis, as well as cell fragments.

Garth Fletcher: You noted an increase in blood sugar levels following treatment. I understand that you acclimated your fish to tanks for 24 hours. Since blood sugar responds readily to such things as handling, are your blood sugar levels at base line levels after 24-h acclimation?

Don McLeay: No; although they have dropped sufficiently to be able to measure a marked hyperglycemia due to effluent exposure. A number of recent studies with both coho salmon and rainbow trout (*Salmo gairdneri*) suggest that 48 h might be a better acclimation period. More prolonged acclimations result in a diminished response to the toxicant (presumably due to depletion of liver glycogen reserves since fish are unfed), even though the control values are somewhat lower.

Garth Fletcher: With reference to the use of blood sugar changes in marine fish I would like to comment that several investigators have shown that some marine fish show greatly elevated glucose levels following capture - the glucose returns to "base line" levels within 3-7 days and is extremely difficult to re-elevate for months following its initial elevation. This lack of response to restressing in marine fish obviously differs from freshwater fish.

Don McLeay: Possibly the lack of a secondary blood sugar response to stress in captured marine fish is due to depletion of their glycogen reserves.

Garth Fletcher: This does not appear to be the case.

Jerry Payne: In the case of your kraft effluent, if there are large sulfite concentrations, would it be possible to do a sulfite oxidase assay? I am just wondering if this enzyme system is inducible.

Don McLeay: There is very little sulfite in kraft effluent, although the concentration of sulfate is quite considerable. The toxic responses in fishes exposed to kraft whole mill effluents are thought to be induced mainly by their resin and fatty acid content.

Gary Vigers: I like the data that you presented on the WBC-T counts. This response is of particular interest in view of the evidence of increased incidences of fish diseases in polluted waters such as the New York Bight. Would you care to comment on the environmental significance of this response?

Don McLeay: Environmental stressors have been shown by numerous investigators to predispose fish to diseases. The depression of the WBC-T counts in salmonid fish due to stress is largely a consequence of the lysis of circulating lymphocytes as a result of increased secretion of corticosteroid stress hormones. Lymphocytes have now been shown to be intimately involved in the immunological competence of fish as in higher vertebrates. As you suggest, a decrease in WBC-T counts in fish due to pollutants might help explain the increased incidences of disease in areas such as the New York Bight.

Ed Sherer: The data you presented on reduction of upper lethal temperature of coho with increasing BKME concentrations were very consistent and convincing, and their ecological importance is obvious. Would you say that "a fish is a fish" and "effluent is effluent"? Have you applied this test to other species?

Don McLeay: I can only say that this test works well with kraft effluent, using either coho salmon or rainbow trout. On one occasion we examined this test with other toxicants (phenol, copper and zinc). Based on their 96-h LC50 values, the temperature tolerance test was less sensitive to these toxicants than to pulpmill effluent.

F.K. Fahmy: I noticed that in most of your experiments you were concerned about the determination of the concentration at which the sublethal effect is significant. Most of the tests were done within hours and in some of them, if you had extended the exposure, the symptoms may have disappeared. Have you ever considered a recovery period or an adaptation period?

Don McLeay: That's a very good point. With regard to the biochemical tests, we have examined responses in fish where exposure to effluent is continued for periods of up to 200 days. Blood sugar levels decrease from an initial marked hyperglycemia to values just slightly above those of controls; and WBC-T counts recover with exposures of 4 days or longer. These responses are consistent with the "stage of resistance to stress" described by Hans Selye for mammals. Therefore recovery of these variables to control levels with prolonged exposures does not necessarily mean that the animal has adapted fully, since the regained regulation observed might require a greater expenditure of energy. We recognize that, as with LC50 bioassays, acute sublethal bioassay tests do not necessarily allow for prediction of "safe" concentrations of these effluents within the environment. The tests described here must be considered together with results from long-term studies such as the "maximum acceptable tolerance concentration" test, other short-term tests measuring responses such as avoidance/preference and olfaction, and exposure-recovery studies, in order to make meaningful estimates of safe discharge limits.

PANEL DISCUSSION

STANDARDIZATION FOR RESEARCH AND REGULATORY STUDIES

PANELISTS

P. DOUDOROFF, OREGON STATE UNIVERSITY
J.B. SPRAGUE, UNIVERSITY OF GUELPH
R.H. COOK, FISHERIES AND ENVIRONMENT CANADA
J.C. DAVIS, PACIFIC ENVIRONMENT INSTITUTE
E. BIRCHARD, IMPERIAL OIL LIMITED
G. LAROCHE, MCGILL UNIVERSITY

INTRODUCTORY ADDRESS BY

P. DOUDOROFF

Dr. Peter Doudoroff of Oregon State University at Corvallis opened the discussion with a talk. What follows is a summary of his remarks. In his comments Dr. Doudoroff was somewhat critical of the directions that have been taken in water pollution research and regulation in recent years. In the early years of bioassays when he started, biologists were interested in standardization largely as an attempt to upgrade the quality of the work, much of which was done by non-biologists and with little regard for important biological considerations.

His main interest, however, was the thought that bioassays often would be a more reliable way of approaching the regulation of waste discharges than strictly chemical criteria. It became apparent that the more one makes the results comparable, the less they become pertinent to specific local situations where the results are to be applied.

At present he sees no need for further standardization of research methods. Too much standardization would result in the missing of a lot of important information. Research tests should be increasingly under, as nearly as possible, natural conditions. The more sensitive sublethal tests interest him, but he suggested we are not sufficiently trying to relate the results from these bioassays to how organisms react in their natural environment. We have to be sure that they are pertinent to what we want to know.

On the other hand, he agreed that standardization of regulatory tests is very important in order to have legal validity. He also agreed with the thinking that we should not be limiting the concentration of toxicants in an effluent without regard to the volume of effluent. He was not enthusiastic about the type of effluent standards that are unrelated to the assimilative capacity of receiving waters. He gave a number of reasons for this point of view; in the first place, these standards often do not provide sufficient protection from sublethal effects when the dilution of the effluent is insufficient. Secondly, they discourage the location of industry in those places which are most advantageous from an environmental standpoint. Thirdly, these standards may overprotect in some cases, with the resulting unnecessary cost to society. Fourthly, they discourage conservation of water by industry.

Lately, Dr. Doudoroff has been interested in the question of the relative value and utility of the chemical criteria of water quality versus the more biological criteria. Some day in the future he believes that we may be able to use largely chemical criteria, but presently we are often too simplistic and inflexible in our choice of effluent standards. Criteria for toxic substances are frequently set without regard to important modifying conditions. Control of waste discharges on the basis of toxicity bioassay results can often be much more reliable than that based on results of chemical analysis, and sound guidelines are needed to direct the choice of most appropriate criteria.

John Sprague: I'd like to donate four and a half of my five minutes to somebody else, but simply say that Peter Doudoroff really knows whereof he speaks. He has taken a small stream in Oregon, injected pollutants into it and studied what really happens in a stream when it is polluted a little bit. He's also put predators and prey in a pond and reduced the dissolved oxygen level to see what effect this had on predation and escapement.

Bob Cook: I've detected from the discussions that standardization is really not that good an approach for research but does have value in the regulatory sense. The scientific method is a good guideline for the research element. A good experiment will also stand on its own merit.

But there are advantages for standardization in regulatory function. Many of us are environmentalists at heart, but are trying to be realistic and fair in our dealings with pollution sources that at times kill our fish. We get a little emotional about it, but we are trying to develop sane strategies. Sometimes the trade-offs are against us; sometimes the trade-offs are against the sources. The approach that Canada has adopted at present is that pollution is to be controlled at the source. I enjoyed Dr. Doudoroff's comments about the chemical measurements moving into a biological measurement and perhaps eventually going back to chemical measurements. Right now we're in that middle stage. We have chemical measurements, some of the rather rough ones like BOD and suspended solids, that are applied at the end of the pipes. Rather than conducting a thousand measurements, we have to use a bioassay procedure. We're trying to establish an LC50 assessment that scientists can relate to when they are talking to operational people. Hopefully the operational people can relate their objectives to the scientific community. This workshop has highlighted where we are getting relationships to LC50's. Once we've got that, everybody's relating back to this common message.

We have a policy of no pollution havens in Canada. This means that there have to be national standards based on best practical technology (BPT). The BPT can be defined as a level of practice that an industry is expected to perform with respect to its treatment. We're going to say that BPT is available, that it is economically sound to apply, and that we expect it as a minimum level of treatment. If those baseline standards are not adequate enough to protect sensitive environments, local authorities will make the requirements more stringent.

John Davis: You've heard a lot of my views on standardization, so maybe I'll just start with some criticisms of what Bob has just said. It's fine to control pollution at the source, but as Dr. Doudoroff has so ably pointed out, this may be a very dangerous precedent if the environment is not considered. We need a combination of approaches. The use of the bioassay procedure rather than thousands of chemical tests is useful, but as Dr. Zitko so ably pointed out, you must consider the chemistry too. We have to have a balance of the two. Biologists cannot work in ignorance of the water chemistry, particularly in such very, very complex situations as aquatic toxicity.

I know that the concept of not encouraging pollution havens in the country is a sensitive one. I've always believed that maybe we could do some sort of limited zoning in managing the region and that we should at least consider the assimilative capacity of certain areas where that capacity could be used. Particularly in British Columbia where we have very long fjord-like inlets for reasons of water use and shipping, we've located industries in absolutely the worst places at the heads of these long inlets which then fill up with pollutants. Then we look at others on well tidal-swept waters and environmental effects on intertidal organisms, for example, are very hard to find. I'm making a plea for considering the assimilative capacity in some way. If regulatory agencies, as they grow in wisdom and good sources of advice, can have at their command a flexible group of criteria and tests and a body who can judge the application of these sorts of rules, we'll have a much more flexible situation without encouraging pollution havens in certain areas.

Evan Birchard: Within industry and certainly within the petroleum industry, we are involved in two basic types of bioassay - routine and research.

On the routine side, our industrial bioassays are run on various effluents, from drilling rigs, refineries, gas plants, etc., that are deposited into receiving waters.

These are fairly standardized bioassays, either 24-h static or 96-h flow-through. Both our own labs and consulting labs that we use attempt to standardize the procedures as closely as possible. The "standardization" basically means that we follow procedures outlined in either the federal regulations or guidelines or the provincial regulations or guidelines. There are problems even in the routine bioassays - when they are conducted for routine monitoring purposes, time is often a very great problem in quickly solving a problem in a plant. Consequently faster test methods have been investigated in various parts of the country. For example, in Canada we have been looking at test methods with rainbow trout. In the U.S. Dr. Cairns at V.P.I. has done a similar thing with *Daphnia pulex*. For our own particular purposes, trout have certain advantages. It's convenient to have one organism for all our labs or consulting labs because that gives me an opportunity to compare various refineries across Canada. We have never really had any basic problems using rainbow trout, such as have been identified at this workshop. We like using rainbow trout because it's a type of fish one can identify with, it's either recreationally or commercially important in many parts of the country and it's surprising how a local town council can identify with rainbow trout.

We are just beginning to get involved in the research bioassay, mainly in relation to the Environmental Contaminants Act and effects of newly marketed chemicals on human health and the environment. The manufacturer of a new chemical has to justify its release if it has any potential of getting into the environment, whether in the manufacturing process or in the marketplace. This kind of testing may involve from a few months to five years. Our company already has begun testing of one chemical, n-methylpyridene.

I had another comment about "pollution havens". Now that we have national refinery regulations, the petroleum industry basically has a policy that any standards that go beyond these minimum standards should be based on a water management approach taking into account assimilative capacity and other factors. If a company has a plant or plans to construct a plant within an area shown to have a low assimilative capacity, there are two choices - either the company has to put in or upgrade its treatment system to meet a local stringent level or else it simply leaves the area and perhaps goes to an area with a higher assimilative capacity. I don't think you'll find the industry disagreeing with that approach. It's a good one.

Another point which Dr. Doudoroff discussed was the toxic emission rate. Petroleum refineries have a toxicity limit based on concentration; however, included within the federal regulations is a dilution allowance which encourages water reduction or water reuse in a plant without actually penalizing the refinery.

One last point is that industry is also involved in sublethal studies. The pulp and paper industry sponsored the work described by B.C. Research this morning. The petroleum industry has sponsored a study in which Dr. Sprague has been involved. From this sublethal work on our refineries, I hope to see some future work on sublethal threshold ranges for various parameters which will let us avoid routine sublethal monitoring and rely more on chemical monitoring. In the long run, I see acute toxicity testing followed by sublethal testing on a fairly routine basis. I'm hoping eventually that we'll then be able to fall back on chemical monitoring.

Gilles LaRoche: I would like to touch a few items which have been brought forth in this interesting workshop.

We have heard that bioaccumulation of toxicants may be an important factor in determining lethal or sublethal responses. To this I would say, not always. Particularly

with substances exhibiting long induction periods, biological responses may not be related to any bioaccumulation or detectable residual.

Reference toxicants were originally suggested as means of evaluation of (a) intra- and interspecific differences in responses; (b) differences of environmental conditions; and (c) differences in biochemical class responses. There should not be just one reference toxicant since there is no such thing as a panacea, but rather reference toxicants, plural, more or less related to the classes of toxicants under test.

I would like to preface this comment with recent information on fresh water analysis. Out of 5500 observations of volatile organics in water, 1296 different substances have been characterized by EPA scientists using a GC mass spectrometer analytical system which could detect concentrations as low as .1 µg of substance per litre. Furthermore, these investigators have suggested that these 1296 different volatile organics represent only 10-20% of dissolved organics, which leaves a probability that as many as 130,000 dissolved organics may be found in fresh water of eventual drinking quality. Before one asks the chemist to assist, one has to establish the biological relevance of the contaminants, natural or otherwise.

I do not condemn the use of tropical or otherwise sturdy fish in the establishment of toxicity levels. However, in the establishment of water quality criteria, severe limitations may be found in the direct application of toxicity data on one or even more species to guidelines for the receiving waters. In addition, compounding toxicity with a natural infestation or even compounding toxicity with a particularly noxious condition may be of great relevance in the establishment of water quality criteria.

Finally, one may assume that, depending on the nature of the toxicants, different metabolic functions will become early targets for impairment in the course of exposure. It would seem appropriate that some of us spend time identifying classes of toxicants which impair particular metabolic functions. In order to use molecular biological systems as endpoints, one will have to understand some of these common metabolic denominators that transcend fish species, invertebrates and even unicellular species. It would not matter which aquatic species are being exposed, providing that more refined biological endpoints have been identified. Dr. McLeay of B.C. Research is working in this direction; however we certainly have to do more to define the affected metabolic functions. Dr. Li's excellent work on tissue culture is certainly closer to an expression of metabolic effects, even though the relationship between external concentrations and those reaching the cells *in vivo* will have to be established. Dr. Freeman has also shown that *in vivo* exposures will be expressed by significant steroidal changes that begin to offer a systematic metabolic reaction to toxicants. In my opinion, with the diversity of approaches presented here, closer association between most disciplines represented might offer the kind of information needed for reliable rapid tests or for realistic water quality criteria.

Keep meeting once a year on these issues. Repeated encounters are often necessary for people to learn new tricks and the great value in adopting an interdisciplinary approach.

Gary Westlake: Some effluents are going to fail whether one uses a stream standard or an "end of pipe" standard. Presumably as the cleanup program progresses this number would decrease. It is more important that we do something decisively at this early stage than spend a great deal of time deciding which technique is to be used.

Menno Speyer: I'm questioning the validity of LC50's altogether in the regulatory sense. The guidelines state that you place fish in this effluent (100% effluent, etc.) for 96 h. If 50% of the fish survive, the effluent passes the test. What happens if all the fish die in hour 98?

Bob Cook: In EPS we use tests that the scientific community suggests are good tests. They have to match the technology that can be achieved and that's where the interface with engineering comes. Once the standard is there and all the fish die in 98 hours, the effluent has still passed the test. If there is a "98 h phenomenon" or something similar, it would be discussed with the environmental engineers and taken into account with their pollution control design. An attempt would be made to recycle or recover the offending contaminant(s).

Menno Speyer: However, strictly in the sense of the law, the effluents pass. So in essence the industries really don't have to do anything about them?

Bob Cook: This is the important question. Pollution control is an effort requiring interfacing with other governments and with industry on these matters. We need a good scientific basis backing us up all the way, pointing out these problems. We have to use standard fish, but our ears are open to all other types of tests that make the use of bioassays more effective.

Peter Doudoroff: I should like to give an additional answer. The situation postulated, where an effluent passes a 96 h test but not a 98 h test, is, of course, very unusual. But we must recognize, also, the fact that an effluent that does not kill 50% of test fish in any such short-term test for acute toxicity can be very harmful in the receiving water when dilution is insufficient. Other tests and restrictions appropriate to the limited assimilative capacity of the water may then be necessary precautions against the hazard.

Ed Pessah: What happens when all the assimilative capacity is used up?

Peter Doudoroff: That is when you put no more wastes there. Instead, industry must be encouraged to locate plants elsewhere, where waters still have the necessary assimilative capacity that can be profitably utilized. This can not be done by imposing uniform effluent standards independent of plant location. At locations that are more favorable with respect to environmental impact, because the assimilative capacity is there, construction, operating and other costs may be relatively high, and a compensatory reduction of waste disposal costs then must be allowed as an incentive.

Gilles LaRoche: The LC50 test, as far as I'm concerned, is not adequate, because we don't know enough about the ecosystems that we're trying to protect. We have to know more. This is why work such as that done by Dr. Li and Dr. Freeman should be sponsored to a greater

extent. Then reliance on the LC50 is perhaps realistic. First we have to establish the vital functions that have been affected. We have to go beyond death. No one here would take a pill that kills half the population, even with an application factor.

John Sprague: I'll take a hundredth of it though.

Gilles LaRoche: Not even a hundredth.

John Sprague: I would.

Cecil Inniss: If I understand the nature of biological time, you will probably not find mortality after 98 h if you did not find it after 96 h. In nine years of testing I have not seen it.

Menno Speyer: I exaggerated the point. Let's say after two weeks?

Cecil Inniss: I've been working on one organic chemical industry and have seen a situation in which fish are alive but spinning slowly on their heads at the end of the test. One would question whether they would survive in the environment. Obviously we must categorize these pollutants. The LC50 test may run into difficulty when used to assess an industry like this. However, since EPS was formed the LC50's of some effluents in Ontario have improved.

Ed Pessah: After the baseline standards are completed, one hazard we have is potential overkill (too much improvement of effluents). When there is underkill the provinces, municipalities and the people must respond. You must take a look at where we've been and where we're going and the need for a type of Band Aid. That's probably what the baseline standards are all about; it's phase one in a multiphase project.

Menno Speyer: You must be sure you are using the right remedy.

Peter Wells: I could add one comment to Menno's point about 4-day LC50's. Quite often, 4-day LC50's are used definitively for comparing the lethal toxicity of pure chemicals. If the LC50's are close to each other, that's a very risky venture. We can get an approximation of how risky it is by plotting toxicity curves using either LC50's or LT50's. Such plotting and comparing of toxicity curves can take some of the error out of comparing 4-day LC50's of substances which may, if the test continued a few days longer, be shown to be equally lethal.

Peter Hodson: I'm addressing Dr. Doudoroff's statement on sulphide. You said that hydrogen sulphide should be regulated when pH is 6 and that the objective, or standards, should not apply when pH is 8 and it is obviously non-toxic and not a problem.

Peter Doudoroff: The recommended criterion is for total sulphide, not for hydrogen sulphide, but it is based on the toxicity of H_2S, which constitutes a varying fraction of total sulphide depending on the pH of the water.

Peter Hodson: Then we could have a situation where a company could release several ppm at pH 8 and not violate the standard. If a company just downstream releases an acid waste, stays within the pH objective but makes the sulphide quite toxic, who do we blame? To paraphrase, should we write our objectives to protect against all situations, or should we write our objectives to protect only against specific situations in which the material is toxic at the point of release?

Peter Doudoroff: Proper control of pollution with substances such as sulphides and cyanides is best achieved through determination of the concentrations in the receiving waters of those particular forms that are actually toxic. It should not be assumed in advance that something else will be added to the water that will increase the toxicity of substances being introduced. Only when this actually happens or can be expected to happen in the absence of appropriate corrective measures must such measures be taken, after determining who is or would be primarily responsible for violation of water quality standards. Although I have emphasized the value of toxicity bioassays of effluents in the control of toxic waste disposal, I believe that chemical tests of receiving waters often are essential and that the bioassays of effluents can sometimes be irrelevant and worthless. An effluent that is not at all acutely toxic can render the receiving water highly toxic even in the absence of any other pollutants; a good example is an effluent that contains certain nontoxic metallocyanide complexes and is diluted with a natural water of much lower pH or is exposed after dilution to enough sunlight, causing liberation of much of the cyanide as highly toxic HCN. We need clear guidelines that would help regulatory agencies and industry choose between the bioassay approach and the chemical approach in dealing with each particular situation or waste disposal problem and would let them know which tests are to be relied upon when the two approaches yield results that do not agree.

Claude Delisle: There are considerable unknowns in laboratory bioassays such as fish acclimation, stress during fish transportation, storage of effluent (risk of biodegradation), exponential decrease of concentration during static tests, contamination of fish food by mercury, etc., unknown and known effects of plastic tubing and containers on fish, genetic variations between different populations of the same species, water quality variation between labs (pH, hardness, etc.), interaction and chelating of a discharge effluent with its receiving water, fish species to be used (flagfish and zebra vs rainbow trout), correlation between laboratories' bioassay results, etc.

I suggest a day or half-day session at next year's meeting on *in situ* bioassays to popularize their utilization and develop better in-field techniques. This will eliminate most of the uncontrollable parameters mentioned above, and by using the most common, sensitive organisms (fish or invertebrate) present in the natural environment, we thus eliminate all the confusion on which species to use, under which conditions, etc. This is proposed as an immediate solution until our laboratory techniques are better defined and in general agreement. The feasibility of doing such *in situ* tests should at least be considered more seriously. The limits of such bioassays are known but at least they inform us on the toxicity of an effluent discharged into the natural environment. It is time to study seriously why we are doing more tests.

Bob Cook: We're living in a world of limited resources. We have a large environment and have to be discretionary with our money for the best protection job. That's why there is a lot of emphasis on the end of the pipe, point source investigations because that's the basis

of our industrial negotiations.

Two things have to be taken into account with respect to the "end of the pipe" concept - is it a new "pipe" where a lot of technology can be applied before the "pipe" is installed or is it an old "pipe" from an industry that has been polluting over the years. In the first case negotiations can be conducted on the location of that pipe so that it can be compatible with the environment, both from a technology and a placement point of view. In the second case, there are financial problems in trying to upgrade industries and they can't be relocated easily. Our major problems lie with the existing industries.

We already have several sets of water quality criteria based upon a lot of data. There are government people from all walks of life going up and down rivers collecting water samples and documenting what levels are. We have some field water quality criteria; we also have a fairly reasonable idea of some of the waste characteristics. It seems we have an information base that can start bringing about environmental improvements just by its use. This precludes getting involved in new work. This is the general rule because specific problems require resources for getting answers. There's a lot of available information in this country and, using the international experience, we should now make some clearer interpretations of it and use it more effectively for pollution control.

Ed Pessah: To quote Dr. Donald Mount, in his opening address to the recent ASTM meeting, "Gentlemen we have a lot of data. Are we making enough use of it?"

Gilles LaRoche: An *in situ* bioassay is an excellent interim procedure. However, more needs to be done to understand the significant toxicological responses which would limit the survival of desirable ecosystems.

In addition, in reference to one industry dumping H_2S and the other one bringing it to an acid pH, we're going to have to resort eventually to a form of land use planning in a very broad sense, allowing dumping in some places by certain industries.

John Davis: I agree that the *in situ* test is one on which we need to do more work. Some of us could raise very sincere objections to some of the procedures used in *in situ* bioassays. Maybe Don McLeay would like to talk about his experiences in trying to do long-term studies of that type. Certainly one can get some rather meaningful indications of whether or not there is a problem actually occurring in the receiving waters. I see them as very useful for that purpose.

I think that we're falling down in this whole business by not coming up with the right sorts of numbers that can be used for water quality criteria. John Sprague really roasted the physiologists a while ago for not producing these numbers in a really good paper at the Pacific Science Congress (Sprague, J.B. 1976. Current status of sublethal tests of pollutants on aquatic organisms. J. Fish. Res. Board Can. 33:1988-1992).

Ron Wallace: I'm a little bit leery about the stated policy of the government of not having pollution havens in Canada. I think we're just a little smug at times; I think that the stated approach is a little bit fallacious at times. What we have is a policy where we don't endorse pollution havens but in fact there are *de facto* pollution havens at the moment. It's true that the philosophical intent is to eliminate and minimize them, but they still exist in a lot of places.

After having been in both a regulatory situation and a research situation, it appears that we came to grips with industry quite a lot of times and not on philosophical

issues. The things we discussed with industry cost a lot of money and at those times discussions got pretty heavy. I always thought that the LD50 approach and associated spinoffs that have come from it were really an extreme answer. The question in my mind was: "What was the extreme question that this extreme answers was being brought up against? The extreme question from industry was why should we spend millions to clean up effluents? And that's often a pretty good question. Usually it's asked by some pretty good engineers and it's difficult to answer.

I think we might be missing the point, in that regulatory agencies in Canada and the United States are at the "front lines" and we have to accept it. They accept, I think, that they are working with pretty extreme answers to pretty extreme questions. The point is that once the industry or the point source is moved beyond the regulatory context through the net, where's the follow-up? We're at the point as toxicologists and biologists that medics were reaching in 1900. They were beginning to recognize the value of preventative medicine but only after they had developed some powerful antibiotics for treatments. Today, we are evolving towards a point of "preventative medicine", i.e. watershed management or resource management in the broad sense. That is where we're falling down. The Environmental Protection Service is out there on the "front lines" fighting the "war" and the occasional problems that get through are simply being let through at the moment. The big management picture on a national and regional basis in Canada is a vacuum at present and it's a vacuum from the policy level at the very top through the whole governmental spectrum. We have people here from a spectrum of agencies and they should be getting together. We can then begin to give some commonsense answers to some commonsense questions over the longterm.

Ed Pessah: Ron, you've partially summarized the concept of the Toxicity Workshop but I chastize you a little bit for stating that regulatory agencies were static and not dynamic. Your net, as it were, seems to have stopped with acute lethal bioassays. You're only looking at Phase 1. What we're talking about here is just what you're addressing now - after Phase 1, what then? Surely by the time Phase 1 is over we've got to have some of the answers and not just the questions.

Gilles LaRoche: In your remarks I would consider, instead of the word extreme, interim - interim answers to interim questions. That is unfortunate and totally unfair. In some instances we proceed with interim answers that will eventually cost everyone (industry and us) millions of dollars. This is why a more comprehensive toxicological program should back up the kind of criteria we derive. I don't think we're doing enough. I must say that I don't like the LC50.

Fahmy Fahmy: You mean the 96 h LC50?

Gilles LaRoche: 96 h or one or two weeks or one hour.

Fahmy Fahmy: But the LC50 by itself? I don't believe that you are against it.

Gilles LaRoche: I'm not against it, but it should not be used to set criteria. It should be used to establish levels at which we are going to begin to test for toxicological responses.

Ed Pessah: We are talking about the same philosophical goals, but I do not see the

mechanisms for achieving those goals. What are the mechanisms for protecting environmental quality?

Gary Westlake: I would suggest a topic for next year based on this discussion - the use of bioassays to solve environmental problems. Assuming that we had 13 papers, at this time next year we might have solved 13 environmental problems, which is a worthwhile goal in itself.

John Davis: If we have agreed that we should carry on with this group and organization in the future, we might devote part or all of these sessions to trying to solve problems. One way of getting everybody involved and developing lists of priorities, approaches and agreements to do combined work is to have a number of criteria or approaches and to break up into discussion groups and really brainstorm these. Then we should have a spokesman for each group presenting the results of these discussions for general involvement and argument. It works very well as long as you keep the groups small and have enough time to get everyone involved. This type of meeting could be a combination of presentation of information as we have been doing, identification of problems, and then further extension to discussion groups.

Gordon Craig: If we're looking at an LC50 or an EC50, you have to measure a response and have to set up concentrations. According to Peter Hodson, if you want to establish a partial response you've got to describe confidence limits, fiducial limits, etc. You've got to describe the flux. If you set up concentrations, the natural, biological variation plays a part in the closeness of those concentrations. If you set up two concentrations which are analytically different but in which the fish respond similarly, have you got one or two concentrations?

We've been talking about a continuous-flow test. There are very few laboratories in Canada that can carry out a continuous-flow test on an enforcement basis. Yet it's a requirement in some regulations. There's got to be an alternative test. Any laboratory can look at a new type of test and compare it with a continuous-flow test. Don McLeay mentioned blood cell counts but how do they relate to acclimation? When is a fish a good fish? These are all very real problems. Otherwise we're going into a bioassay with tools that have to be tested more than they have in the past and it takes a lot longer to do the bioassay.

For the next workshop, let us look at our tools - what we can do with them and what are their limitations?

Cecil Inniss: If a gentleman says his *Daphnia* test works, he should demonstrate it. If a gentleman says that *in situ* bioassay works, I'd like to see photographs of his net and statistical validation of his data. If someone is going to suggest a method, he should support it and, if possible, compare it to a standard or recognized method.

Bob Cook: I fully endorse the idea of splitting into smaller groups and coming up with recommendations so that the main group can look at them and see where they're going. That's certainly an approach for the next workshop.

Peter Doudoroff: I want to remark that the 96 h lethality test often is not a toxicity bioassay sensitive enough for our needs, but other bioassays that have been proposed, which measure sublethal responses, are very sensitive to some toxic pollutants and much less sensitive to others. I believe that it would be best to rely not on any one kind of

bioassay but on a standardized group of tests measuring various sublethal responses of apparent ecological importance. Chemical tests and standards can be most satisfactory when enough is known about the toxicity and interactions of all the toxic components of a particular waste, and it appears that such understanding of the toxicity of pulp mill wastes perhaps has been approached. Finally, I want to say that I have been concerned about the number of variations of standard bioassay methods that have been proposed by different groups of biologists. The unnecessary publication of somewhat different methods recommended as standard methods by these groups does not, of course, promote true standardization.

Ed Pessah: I'd like to close the sessions officially now.

John Davis: We will try to organize the meeting in Vancouver next year. We hope to get some support from several agencies so as to make the meeting a little bit broader. I'd just like to know (1) is that a good location for people? (2) should we carry on with two day or can we make three day sessions? If anyone has any suggestions on content, please see me and we'll try to put something useful together.

Ed Pessah: People feel that we should continue on with these workshops. We invited speakers whom we felt had a grasp of the area that we invited them to talk about. We minimized the contributed papers so as to maximize the time spent to discuss those papers, discuss the methods and have the discussion that we had this afternoon. Whether we succeeded or not is up to you to decide. Thank you all very much for coming to Halifax for this year's toxicity workshop, and see you next year.

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